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14. ABSTRACT Acute alcohol intoxication (AAI) impairs the hemodynamic countereulatory response to trauma and hemorrhagic shock (HS), blunts the pressor response to fluid resuscitation (FR), suppresses the HS-induced neuroendocrine response, impairs pro-inflammatory cytokine expression and increases mortality from infection during recovery. Studies conducted during this funding period examined a) whether the attenuated neuroendocrine response, particularly reduced sympathetic nervous system (SNS) activation, is the principal mechanism responsible for the hemodynamic instability seen in AAI+ HS and b) what the impact of AAI was on the integrity of host defense mechanisms during the immediate and delayed recovery from HS. We determined whether SNS activation can be restored by central (intracerebroventricular; ICV) choline administration and whether this in turn is capable of improving the hemodynamic countereulatory response to HS in AAI. Our results show that ICV choline stimulates SNS activation and restores the initial AAI-induced decrease in basal MABP but did not ameliorate the hypotensive response to 50% blood loss in AAI animals. In addition, our results show that at 1 day, inflammatory responses are suppressed in cells obtained from AAI+HS animals. In contrast, at 5 days cells obtained from AAI+HS animals show enhanced pro-inflammatory responses.					
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INTRODUCTION: The incidence of traumatic injury in alcohol-intoxicated individuals has continued to escalate during recent years. Traumatic injury is responsible for the greatest number of years of potential life lost before age 65; higher than that attributed to cancer, heart disease and HIV. Approximately 25% of injuries treated in emergency departments are alcohol related. Alcohol-intoxicated injured victims are likelier to present with more severe injury. Although improved resuscitation of trauma patients has dramatically reduced immediate death from hemorrhagic shock, the outcome of these patients continues to be complicated with infections and secondary loss of organ function during the recovery period. Frequently patients have to go through surgical procedures to stabilize them or save their lives. These additional invasive procedures add to the risk of complications in the victims of traumatic injury. We have obtained data from our studies showing that alcohol-intoxication interferes with the body's response to loss of blood and in addition, interferes with the restoration of blood pressure with intravenous fluid resuscitation. In addition, we have also observed that the mortality following traumatic injury in the intoxicated host leads to greater mortality from infection. One of the possible aspects that may be affected by alcohol is the brain's ability to adequately activate all of the responses that are necessary to control blood pressure and to ensure that blood flow and oxygen reach all the tissues. Our studies investigate the pathways that alcohol affects leading to inability to restore blood pressure. The studies use an animal model to investigate what are the brain responses that alcohol affects and to see whether restoring these responses will aid in recovery from hemorrhagic shock. The importance of these studies is so that physicians working in an emergency department can immediately recognize that the alcohol-intoxicated patient that comes in with injury and losing blood will respond differently to the interventions that they implement. It will help us understand how to treat these patients differently and improve their outcome. This will reduce cost of medical care, improve survival of the victim and prevent further complications that may delay reincorporation of the individual into the working force.

Progress report 1st funding period:

Studies conducted during this funding period have addressed aspects from each of the three specific objectives proposed. The PI would like to highlight the fact that the studies performed during this first year of funding were the first animal experiments to be conducted in our laboratory since the return to our building following Hurricane Katrina. Despite the difficulties in getting back to our laboratories and replacing all reagents and resources lost, we have made significant progress in accomplishing our objectives. Because of logistical benefits we have performed studies that fall under the 3 objectives of the proposal. Progress made in these will be described accordingly.

Objective 1: To test the hypothesis that acute alcohol intoxication alters central activation of descending sympathetic outflow. The proposed studies will identify the mechanisms responsible for the impaired hemodynamic counterregulatory response to blood loss in the alcohol-intoxicated host. Specifically, to isolate central and peripheral regulatory mechanisms disrupted during alcohol intoxication.

- a. Determine whether direct central activation of sympathetic outflow restores catecholaminergic and hemodynamic responses to hemorrhagic shock in alcohol-intoxicated animals.
- b. Determine whether inhibition of central sympathetic activation during alcohol intoxication is mediated through enhanced tonic inhibition by nitric oxide.
- c. Examine whether central administration of arginine vasopressin enhances sympathetic activation and restores catecholaminergic and hemodynamic responses to hemorrhagic shock in alcohol-intoxicated animals.

Progress: Objective 1a and 1c.

Short-term central activation of descending sympathetic outflow does not restore alcohol-induced hemodynamic instability during hemorrhagic shock. One of the most critical determinants of outcome within the first 48 hours of injury is the victim's mean arterial blood pressure (MABP) at the time of admittance into the emergency department. Previously we have demonstrated that acute alcohol intoxication (AAI) suppresses MABP throughout hemorrhagic shock (HS), decreases blood loss required to achieve hypotension and blunts the pressor response to fluid resuscitation. We hypothesized that attenuation of neuroendocrine and sympathetic nervous system (SNS) responses to HS plays a significant role in the accentuated hemodynamic instability in AAI animals. We investigated whether choline, a precursor of acetylcholine (ACH), would improve hemodynamic stability in AAI animals. Chronically-catheterized, conscious male Sprague-Dawley rats received a primed (1.75 g/kg) continuous (250-300 mg/kg/h) 15-h intragastric (IG) infusion of 30% alcohol or isocaloric/isovolumic dextrose. A second IG alcohol bolus was administered 30 min prior to intracerebroventricular (ICV) choline (150 μ g) injection. Animals underwent fixed-volume (50%) HS and fluid resuscitation (FR; 3X blood volume removed), 15 min after ICV choline administration. AAI (174 ± 13 mg/dL) decreased basal MABP (-15%), accentuated the initial drop in MABP (-20% at 15 min) and prevented restoration of MABP at the end of fluid resuscitation (all $p < 0.05$).

ICV choline increased basal MABP (+17%) and produced a similar increase in basal MABP in AAI animals as shown in figure 1. However, ICV choline did not alter the initial % decrease in MAP nor did it improve MABP throughout HS or FR in alcohol-treated animals, as shown in figure 2.

To determine if ICV choline increases SNS activation, we measured catecholamine levels (Figures 3 & 4). ICV choline produced a marked increase in baseline epinephrine (198%), norepinephrine (76%) and arginine vasopressin (145%) which was abolished in ALC-intoxicated animals as shown in figure 3. These results suggest that ICV choline results in immediate stimulation of SNS outflow, which does not appear to be sustained sufficiently to improve MABP response to HS in AAI.

Currently we have initiated studies that will examine if combined ICV administration of acetyl cholinesterase inhibitors (neostigmine) and choline will improve hemodynamic instability in AAI HS animals through enhancing the availability of acetylcholine in the CNS throughout the duration of the HS period.

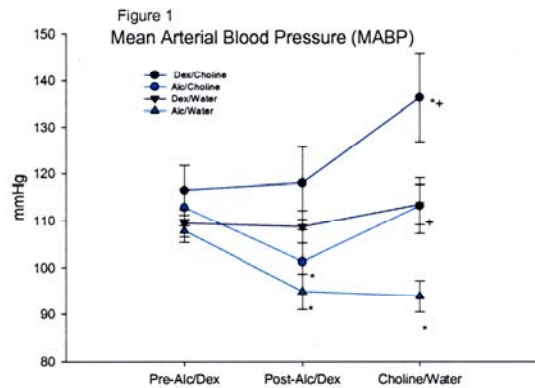


Figure 1. Mean arterial blood pressure response to alcohol treatment and to ICV choline administration.

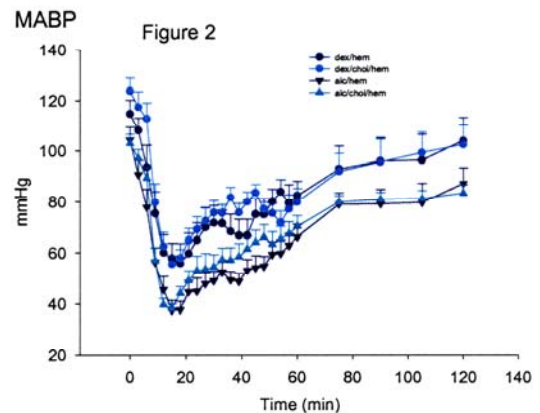


Figure 2. Mean arterial pressure as a function of time of hemorrhagic shock (0-60 min) and during fluid resuscitation (60-120 min) in dextrose and alcohol treated animals that were injected with ICV choline or water.

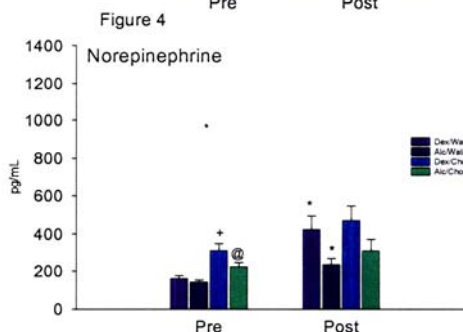
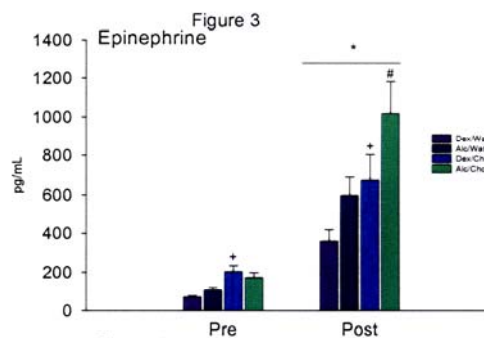
Objective 2: Examine the impact of alcohol intoxication on vascular responsiveness to pressor agent administration.

- To determine the impact of acute alcohol intoxication during trauma/hemorrhage on vascular responsiveness to in vivo administration of pressor agents (norepinephrine and arginine vasopressin).
- To examine the impact of alcohol on vascular reactivity to direct application of pressor agents to isolated vessels.

Progress: objective 2b

Alcohol affects aortic vasoconstrictive and vasodilatory responses. Studies were performed to establish the impact of alcohol alone on vascular reactivity to the direct application of pressor agents to isolated vessels. Male Sprague-Dawley rats were surgically implanted with vascular and gastric catheters. Following recovery from surgical procedures, animals were randomized to receive one bolus of alcohol (5 g/kg; 30% w/v) or 4 days of alcohol (alcohol-binge). Time-matched controls received equal volumes of isocaloric dextrose. Animals were sacrificed 2.5 hours following alcohol administration and the thoracic aorta was removed. This time was chosen in order to most closely mimic the time elapsed from time of alcohol administration and the end of the HS protocol to be used in the studies. A 2 millimeter section of thoracic aorta was hung (4 rings were obtained from each animal) in the Myobath II 4-channel Tissue Bath System from Kent Scientific in a Krebs's Ringer Bicarbonate solution pH set to 7.4, gassed with 95% O₂ and 5% CO₂. Passive tension (stretch; 1.0 gram) was elicited on the vessel rings and they were allowed to equilibrate for 20-30 minutes. Rings were then exposed to 160 ul of a 3M KCl solution and the ability of the rigs to contract was examined. Rings that did not contract at this point were eliminated from the protocol. After obtaining measurements of contraction, the chambers containing the rings were flushed and allowed to restabilize. Subsequently, a dose response curve (10⁻⁹ to 10⁻⁵ M) to Phenylephrine was performed by recording the developed tension. Finally, using a similar approach, a dose response curve (10⁻⁹ -10⁻⁵ M) to Acetylcholine was established to assess vascular relaxation.

Initial studies were aimed at isolating the effects of alcohol on vascular reactivity. Results obtained, shown in figure 5 suggests that aortic rings obtained from alcohol-treated animals had greater responsiveness to the vasoconstrictive effects of phenylephrine and attenuated relaxation response to acetylcholine.



Figures 3 & 4. Plasma levels of epinephrine and norepinephrine obtained before hemorrhage (pre) and at completion of hemorrhagic shock (post) in alcohol and dextrose treated animals injected with ICV choline or water. Values are mean±SEM. *p<0.05 vs. time-matched controls. +p<0.05 vs. pre, @p<0.05 vs. dextrose.

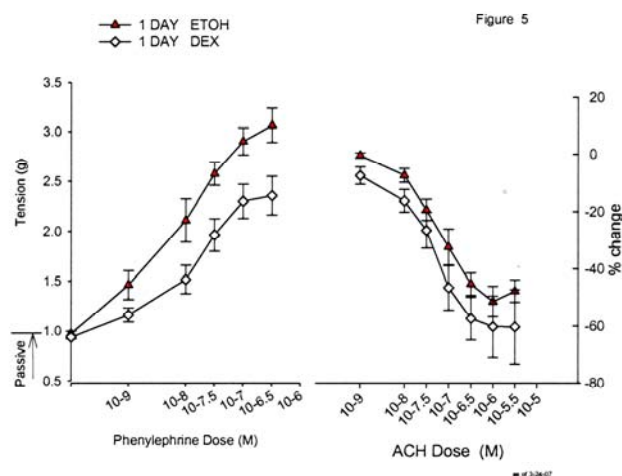


Figure 5. Dose-response tension developed by isolated aortic rings exposed to phenylephrine (vasopressor) and acetylcholine (vasodilator). Aortic rings isolated from alcohol and dextrose treated animals.

In subsequent studies, using a similar protocol as that described above, alcohol was added to the buffer when performing the in vitro studies. Alcohol concentrations in the buffer were matched to those obtained in vivo following the dose of intragastric alcohol used in the studies (150 mg/dl). Buffer Ph was adjusted as needed. Studies were performed with and without alcohol in the buffer in duplicate. Results shown in figure 6 are not definitive as to an effect of alcohol. While alcohol in the buffer appears to suppress vasoconstriction in response to phenylephrine in control vessels, this effect is abrogated in alcohol-treated animals, suggesting an adaptive response. No apparent differences in vasodilatation in response to acetylcholine were detected. Although it would appear that there is a tendency for alcohol treated rings to have a greater vasodilatory response to acetylcholine than those rings obtained from control animals without alcohol in the buffer.

Subsequent studies were performed in animals exposed to a 4 day binge alcohol protocol. Rats were administered an intragastric bolus of alcohol (5 g/kg; 30% w/v) or isocaloric dextrose solution for 3 consecutive days, followed by a 2.5 g/kg dose on day 4. 2.5 hours after alcohol injection, the animals were sacrificed and the tissues were removed.

The results from those studies (Figure 7) suggest that alcohol-treated animals had a blunted vasoconstrictive response to phenylephrine than those that were treated with dextrose. Furthermore, it appears that the vasorelaxation response to acetylcholine is further enhanced in alcohol-treated animals. This protocol of alcohol administration preceding hemorrhagic shock will be used in experiments conducted in the second year of funding.

To determine whether alcohol in the buffer during the in vitro studies would affect this response to vasoconstrictors and vasodilators, a set of studies was performed following the same protocol as described above. The results from these studies are preliminary in nature (N=2-3/group). The preliminary results are shown in figure 8.

Research Objective 3: To test the hypothesis that the alterations in hemodynamics produced by acute alcohol intoxication during trauma-hemorrhage result in inadequate tissue perfusion during the resuscitation period leading to enhanced susceptibility to tissue injury.

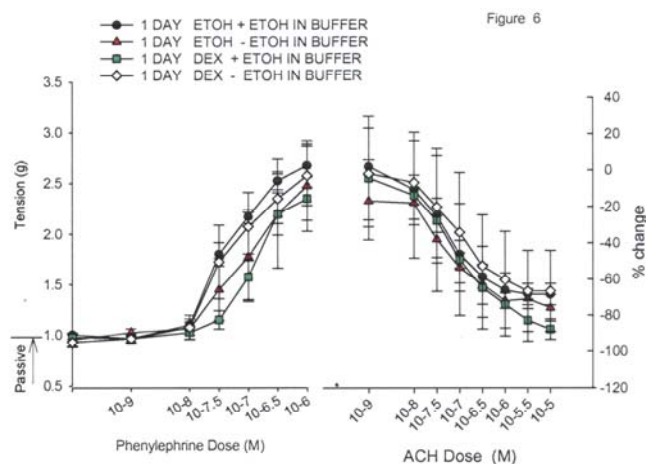


Figure 6. Dose-response tension developed by isolated aortic rings exposed to phenylephrine (vasopressor) and acetylcholine (vasodilator) +/- alcohol in vitro. Aortic rings isolated from alcohol and dextrose treated animals.

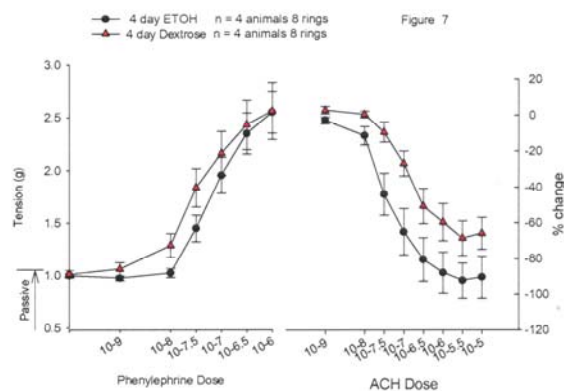


Figure 7. Dose-response tension developed by isolated aortic rings exposed to phenylephrine (vasopressor) and acetylcholine (vasodilator) Aortic rings isolated from alcohol and dextrose treated animals. Note that animals received alcohol 4 days prior to study.

- Examine the impact of acute alcohol intoxication on tissue blood flow redistribution following fluid resuscitation.
- Identify the host defense mechanisms affected by alcohol intoxication & traumatic injury that impair the ability to effectively respond to a "second hit" infectious challenge.

Progress Objective 3b.

Previously, we demonstrated that acute alcohol intoxication prior to hemorrhagic shock impairs hemodynamic and neuroendocrine counterregulation, suppresses early lung pro-inflammatory cytokine expression and increases mortality from infection during recovery. We examined the impact of a 3-day alcohol binge on host responses during trauma/hemorrhage (Tx/hem) and following overnight recovery. Chronically-catheterized adult male Sprague-Dawley rats were administered an intra-gastric bolus of alcohol (5g/kg ; 30% w/v) or isocaloric dextrose solution for 3 consecutive days followed by a 2.5 g/kg dose on day 4 prior to undergoing full-thickness muscle-crush and fixed-pressure (40 mmHg) hemorrhage (Tx/hem) and fluid resuscitation (2.4X total blood volume removed).

Alcohol (BAC = 165 ± 7 mg/dL) produced a 16% decrease in basal mean arterial blood pressure (MABP), decreased the total blood loss required to reach and sustain MABP of 40 mmHg and markedly blunted the rise in circulating epinephrine and norepinephrine (20-fold and 3-fold respectively) levels. Overall, alcohol-treated animals had higher mortality during the fluid resuscitation period (Figure 9).

Consistent with our previous reports, significant upregulation in lung and spleen tumor necrosis factor (TNF)- α and interleukin -1 (IL-1) expression was observed immediately following hemorrhage & fluid resuscitation. Only the tx/hem-induced rise in lung TNF- α was prevented by binge alcohol administration. Following overnight recovery, significant LPS-stimulated release of TNF α , IL-1 α , IL-6, and IL-10 was observed in cells isolated from blood, alveolar and pleural compartment from all experimental groups. While Tx/hem did not prevent LPS-induced release of TNF α , IL-1 α , IL-6 or IL-10 at 6 or 24 hours, alcohol-binge suppressed TNF- α , IL-1 and IL-6 release, without altering IL-10 response in cells isolated from blood and pleural compartment. No significant modulation of alveolar macrophage response was observed following alcohol-binge and tx/hem. These results indicate that 3 day alcohol-binge results in hemodynamic instability associated with attenuated neuroendocrine activation during Tx/hem as well as sustained suppression of the pro-inflammatory cytokine response of blood and pleural-derived cells to an in-vitro LPS challenge. As a result, we speculate that the net shift towards an anti-inflammatory state may

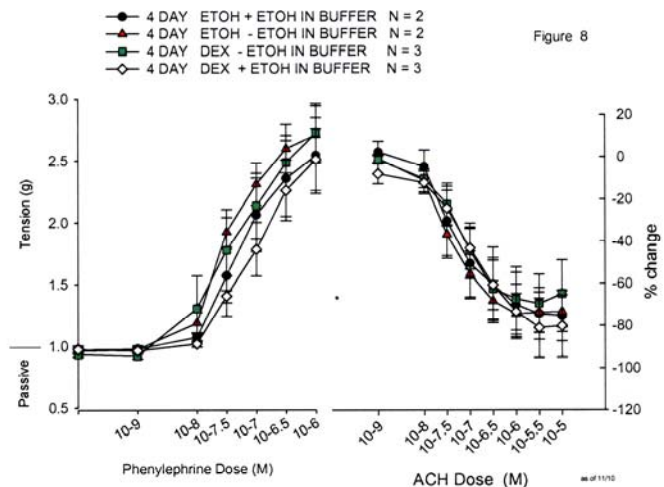


Figure 8. Dose-response tension developed by isolated aortic rings exposed to phenylephrine (vasopressor) and acetylcholine (vasodilator) +/- alcohol in vitro. Aortic rings isolated from alcohol and dextrose (4 day) treated animals.

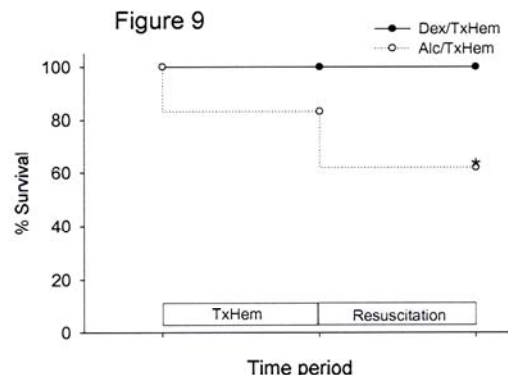


Figure 9. Mortality during trauma/hemorrhage and fluid resuscitation in dextrose- and alcohol-treated animals.

contribute to enhanced susceptibility to infection during the recovery period.

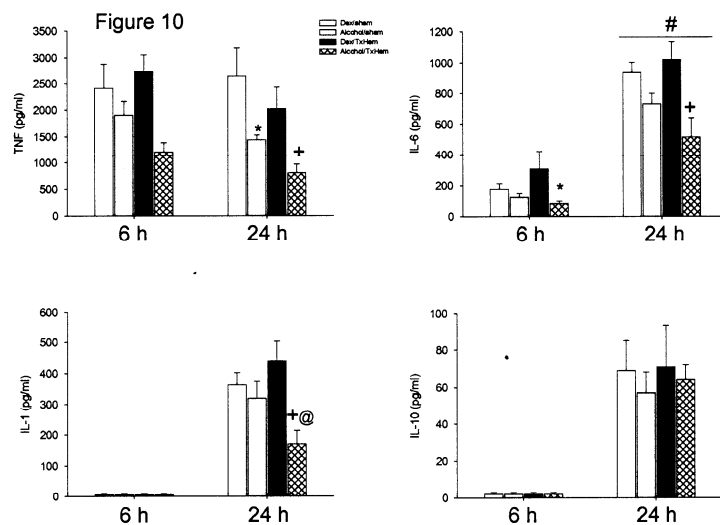


Figure 10. Concentrations of TNF, IL-1, IL-6 and IL-10 measured in supernatants of PBMC isolated from alcohol- and dextrose-treated hemorrhaged and sham animals, stimulated with LPS. Values reflect concentrations at 6 and 24 h of incubation with LPS. * $p < 0.05$ vs. time-matched controls. # $p < 0.05$ vs. 6 h, @ $p < 0.05$ vs. dextrose-treated hemorrhaged and + $p < 0.05$ vs. alcohol sham.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that alcohol intoxication increases mortality from trauma/hemorrhage
- Established the feasibility of using ICV choline to activate sympathetic outflow and increase blood pressure
- Determined that alcohol intoxication decreased blood pressure and blunts the choline-induced rise in norepinephrine
- Demonstrated that ICV choline does not improve hemodynamic response to hemorrhage
- Observed that the activation of the SNS by ICV choline does not appear to be sustained throughout the duration of the hemorrhage
- Determined that alcohol intoxication during hemorrhage blunts host defense response to a second-hit challenge in PBMCs even after a 24 h recovery
- Demonstrated that alcohol intoxication in vivo alters vascular reactivity to pressor agents
- Demonstrated that alcohol in the environment of the vessels affects their ability to respond to vasopressor and depressor effects

REPORTABLE OUTCOMES:

Publications:

1. Mathis KW, Zambell K., Olubadewo JO, Molina, PE. Altered hemodynamic counter-regulation to hemorrhage by acute moderate alcohol intoxication. *Shock* 26(1):55-61, 2006.
2. Greiffenstein P, Mathis KW, Vande Stouwe C, Molina PE. Alcohol binge prior to trauma/hemorrhage impairs integrity of host defense mechanisms during recovery. *Alcohol Clin Exp Res.* 31(4):704-15, 2007.
3. Greiffenstein P, Molina PE. Alcohol-induced alterations on host defense following traumatic injury. In preparation.

Presentations:

- Alcohol binge suppresses early lung TNF expression following trauma and hemorrhagic shock. KW Mathis, K Zambell, J Olubadewo, PE Molina. FASEB, San Francisco, April 2006.
- Alcohol intoxication impairs counterregulatory responses during hemorrhagic shock. KW Mathis and PE Molina. Louisiana Academy of Sciences, Southern University, Baton Rouge, LA (March 2007)
- Acute alcohol intoxication does not modulate cardiovascular and neuroendocrine response to immobilization stress. KW Mathis, Z Zhang, P Greiffenstein, PE Molina. Research Society on Alcoholism, Baltimore, MD, June 2006.
- Alcohol-binge prior to trauma-hemorrhage impairs lipopolysaccharide-induced peripheral blood mononuclear cell inflammatory response. Greiffenstein P, Vande Stouwe C, Williams-Mathis K, Molina PE. Alcohol and Immunology Research Interest Group (AIRIG) Nov 17 2006- Loyola University Medical Center, Maywood, IL.

- Trauma-Hemorrhage Following Alcohol-Binge: Compartment-Specific Immunosuppression.
Greiffenstein P, Vande Stouwe C, Williams-Mathis K, Molina PE. Cohn-Rives Visiting Professorship
Resident Research Day. March 16, 2007- LSU Dept of Surgery, New Orleans

CONCLUSION: The results from the studies performed during this funding period have provided evidence of a marked alteration in the ability of the host defense response to fight an inflammatory challenge during the early recovery period from traumatic injury in alcohol-intoxicated animals. The results also show a greater mortality during trauma hemorrhage and fluid resuscitation when alcohol is on board. Our studies also indicate that while choline administered into the brain produce immediate activation of a sympathetic response and rise in blood pressure this is not sufficient and prolonged enough to restore blood pressure in the alcohol treated animals. These animals then end up with much lower blood pressure levels at the end of fluid resuscitation and this presentation has been clinically shown to be associated with a detrimental outcome from injury. Current studies are investigating alternative mechanisms of ensuring a sustained activation of sympathetic outflow to enhance presser responses to fluid resuscitation in alcohol intoxication. The results obtained from in vitro aortic rings show distinct effects of one day and 4 day exposure to alcohol in vivo and furthermore, suggest a direct effect of alcohol on vascular response that is independent from neuroendocrine activation. These studies are helping us understand whether the limiting factors in restoring adequate blood pressure and perfusion pressure are limited to impaired neuroendocrine activation or if in addition, there are alterations in the ability of the vessels to respond to vasopressors when they are in the presence of alcohol. Taken together, the results from the studies provide better understanding of the pathophysiology that precludes optimal resuscitation of the alcohol-intoxicated trauma victim.

APPENDICES:

2 Manuscripts published.

ALTERED HEMODYNAMIC COUNTER-REGULATION TO HEMORRHAGE BY ACUTE MODERATE ALCOHOL INTOXICATION

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Received 17 Nov 2005; first review completed 1 Dec 2005; accepted in final form 27 Jan 2006

ABSTRACT—The incidence of traumatic injury, frequently associated with hemorrhagic shock, is higher in the alcohol-intoxicated individual. The outcome, as it pertains to both morbidity and mortality of this population, is partly dependent on duration of alcohol exposure and levels of blood alcohol at time of injury. In previous studies, we demonstrated that prolonged alcohol intoxication (15-h duration) produces marked hemodynamic instability and exacerbated early lung proinflammatory cytokine expression after hemorrhagic shock. The present study examines whether a shorter and more modest period of alcohol intoxication is sufficient to alter hemodynamic and proinflammatory responses to hemorrhagic shock. Chronically instrumented, conscious male Sprague-Dawley rats (250–300 g) received a single intragastric bolus of alcohol (1.75 g/kg) 30 min before the administration of fixed-volume (50%) hemorrhagic shock, followed by fluid resuscitation with Ringer lactate. Time-matched controls were administered on isocaloric dextrose bolus (3 g/kg). Alcohol (blood alcohol concentration, 152 ± 10 mg/dL) produced a 14% decrease in basal mean arterial blood pressure and a more profound hypotensive response to equal blood loss. The 2-fold rise in circulating norepinephrine levels was similar in alcohol- and dextrose-treated hemorrhaged animals despite greater hypotension in alcohol-treated animals. Significant upregulation in lung and spleen interleukin (IL) 1, IL-6, IL-10, and tumor necrosis factor α expression was observed immediately after hemorrhage and fluid resuscitation, as previously reported. Only the hemorrhage-induced rise in lung IL-6 and tumor necrosis factor α was prevented by alcohol administration. In contrast, spleen cytokine responses to hemorrhage were not altered by alcohol administration. These results indicate that moderate acute alcohol intoxication results in significant modulation of hemodynamic and neuroendocrine responses to hemorrhagic shock.

KEYWORDS—Ethanol, blood pressure, catecholamines, corticosterone, cytokines, rats

INTRODUCTION

Alcohol intoxication and abuse contributes to a third of all traumatic injury deaths each year, including motor vehicle accidents, accidental falls, sports injuries, burn, and firearm injuries (1, 2). A significant number (nearly 50%) of all injured patients in emergency rooms have positive blood alcohol concentrations (BACs), and many of these have levels above 100 mg/dL, the legal limit of intoxication in most states (3–6). Injured and intoxicated patients have greater risks of mortality, a greater need for intensive care, and are more likely to develop permanent disabilities and acute medical complications such as pneumonia, sepsis, and other life-threatening infections (7). Among the possible confounding factors for these complications is the severity of the injury, genetic makeup of the individual, the history of alcohol intake of the individual (acute intoxication versus chronic alcohol consumption), the total amount and duration of alcohol exposure before the traumatic event, and the concentration of alcohol present in the blood (4, 8).

Previous studies (9) from our laboratory have shown that the hemodynamic, neuroendocrine, and immune responses to fixed-pressure (mean arterial blood pressure [MABP], approx-

imately 40 mmHg) hemorrhagic shock are markedly affected after a prolonged (15 h) period of alcohol exposure, achieving blood alcohol levels of 190 ± 21 mg/dL. In subsequent studies (10), we demonstrated that these alcohol-induced alterations were associated with greater morbidity and mortality from a subsequent infectious challenge. Whether shorter and more modest alcohol intoxication would produce similar alterations in the counter-regulatory responses to hemorrhagic shock was unclear from those studies. Thus, the aim of the present study is to examine the impact of acute 30-min alcohol exposure on hemodynamic, neuroendocrine, and immune responses to fixed-volume hemorrhage.

MATERIALS AND METHODS

Animal preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center and were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health. Specific-pathogen-free adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 250–300 g were housed in the Division of Animal Care at Louisiana State University Health Sciences Center for at least 1 week before the performance of the experimental procedures to acclimate them to their surroundings. The animals were fed standard rat chow (Purina, Richmond, Ind) and housed in a controlled environment at a temperature of 22°C with 12-hour light/dark cycle exposure. Two to three days before alcohol administration and hemorrhagic shock, the animals were anesthetized with ketamine/xylazine (90 mg/kg and 9 mg/kg, respectively) and implanted with vascular (carotid and jugular) and gastric sterile catheters using aseptic surgical procedures as previously described by our laboratory (9). The catheters were routed subcutaneously through a trocar and exteriorized at the nape of the neck through a 1-cm incision. After surgery, the animals were placed into individual cages and allowed to completely recover from anesthesia before providing them with food and water

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ad libitum. All experimental procedures were conducted in conscious and unrestrained animals and were initiated at approximately 7:30 am.

On the day of the study, the carotid artery catheter was connected via a pressure transducer to an amplifier that relayed the signals to a data acquisition system (PowerLab; ADInstruments, Inc, Colorado Springs, Colo). MABP was monitored throughout the duration of the experiment, and recorded and averaged every 3 min. Animals were randomly divided into 4 groups: (1) alcohol-treated, not hemorrhaged (alcohol/sham); (2) dextrose-treated, not hemorrhaged (dextrose/sham); (3) alcohol-treated, hemorrhaged (alcohol/hemorrhage); and (4) dextrose-treated, hemorrhaged (dextrose/hemorrhage).

Alcohol and dextrose administration

Animals received a single intragastric 30% alcohol (1.75 g/kg) bolus 30 min before the hemorrhagic shock protocol. Time-matched control animals were administered isocaloric 52% dextrose (3.04 g/kg). The blood alcohol levels obtained with the single intragastric bolus are consistent with BAC commonly seen in injured patients in emergency rooms across the United States (3). The alcohol administration protocol used was selected to reflect the levels of alcohol intoxication achieved in humans after intake of approximately 2 to 3 drinks, where each drink has approximately one-half ounce of absolute alcohol. Additionally, intragastric administration of alcohol was selected for these experiments, as it most resembles the route of alcohol intake in humans.

Hemorrhagic shock and fluid resuscitation

At 30 min after alcohol or dextrose administration, 50% of the animals' estimated total blood volume was removed over a 60-min period, with 40% removed within the first 10 min of hemorrhage and the remaining 10% removed at different intervals throughout the hemorrhage. The arterial blood removed was collected into a heparinized syringe. Animals were not heparinized before, during, or after hemorrhage. At the end of the 60-min hemorrhage, the animals received an intravenous bolus of warmed (30°C) Ringer lactate to provide 40% of the total blood volume removed. This was followed by a constant intravenous infusion of Ringer lactate (approximately 20 mL over 60 min) to provide twice the total blood volume removed. In total, 2.4 times the blood volume removed was replaced with Ringer lactate. MABP was averaged and recorded every 15 min throughout the resuscitation period.

At the end of fluid resuscitation ($t = 150$ min), the animals were euthanized by an intravenous injection of sodium pentobarbital (125 mg/kg), followed by exsanguination. Tissues (lung and spleen) were excised immediately, washed in normal saline, freeze-clamped with liquid nitrogen, and stored at -80°C .

Analytical procedures

Arterial blood samples were collected in chilled heparinized syringes, and aliquots were placed in tubes containing aprotinin (Sigma, St Louis, Mo) at

10 $\mu\text{L/mL}$ of blood, or catecholamine preservative (9% EDTA, 6% glutathione, and dH_2O at a pH value of 6.0–7.4) at 20 $\mu\text{L/mL}$ of blood. Blood samples were centrifuged for 15 min at 10,000 rpm for plasma separation. BACs were measured using an amperometric oxygen electrode (Analox Instruments Ltd, London, UK). High-performance liquid chromatography was used to quantify circulating epinephrine and norepinephrine levels. Plasma samples were spiked with 30 μL of 3,4-dihydroxybenzylamine, the internal control, and absorbed into a small quantity of aluminum oxide (alumina). The samples were quantified for circulating epinephrine and norepinephrine levels using a high-performance liquid chromatography system consisting of a chromatographic analyzer with a catecholamine column and an electrochemical detector (Bioanalytical Systems, Inc, West Lafayette, Ind). Corticosterone levels were measured as an index of activation of the hypothalamic-pituitary-adrenal axis, a hallmark of the stress response, using a rat-specific radioimmunoassay (DPC; Diagnostic Products Corporation, Los Angeles, Calif). Lung and spleen proinflammatory cytokines, IL-1 α , IL-6, and TNF- α , and the anti-inflammatory cytokine IL-10, were determined using rat-specific enzyme-linked immunosorbent assays (Biosource International, Camarillo, Calif) and expressed as units per milligram of protein. Myeloperoxidase (MPO) activity was determined as an index of neutrophil infiltration using a modified enzymatic approach (11). Briefly, tissues were homogenized in buffer (100 mg/mL of 20 mmol/L phosphate/0.1 mmol/L EDTA, pH 7.4) and homogenates were subjected to centrifugation (10,000g for 5 min) at 4°C . The supernatant was discarded and the pellets were immediately frozen in liquid nitrogen. Pellets were resuspended in 3 mL buffer (50 mmol/L phosphate buffer/0.5% hexadecyltrimethylammonium bromide, pH 6.0), sonicated for 10 min, subjected to 2 cycles of freeze-thaw, and sonicated again before a final centrifugation step (20,000g for 15 min) at 4°C . MPO activity in supernatants was assessed by measuring the change in absorbance at 450 nm (Ultraspec; Amersham Pharmacia Biotech, Piscataway, NJ) in 150 μL reaction buffer (80 mmol/L phosphate-buffered saline solution, pH 5.4, containing 1.5 mmol/L H_2O_2 and 1.6 mmol/L 3, 3', 5, 5'-tetramethylbenzidine) at room temperature. The reaction was stopped with 50 μL of 3 mol/L H_2SO_4 (pH 3.0). Under these conditions, 1 unit of MPO activity produces an increase in absorbance of around 5 per min, resulting from decomposition of H_2O_2 and oxidation of tetramethylbenzidine. MPO activity (units/g per minute) is expressed as the amount of enzyme necessary to produce a change in the absorbency of 1.0 per minute per gram of tissue.

Tissue preparation

Frozen tissue samples were weighed and placed in a homogenization buffer at 4°C (100 mg tissue/1 mL of buffer). The homogenization buffer consisted of a protease inhibitor solution containing 1 mmol/L phenylmethylsulfonyl fluoride (Bachem California Inc, Torrance, Calif), 1 mg/mL pepstatin A (Bachem California Inc), and 1 mg/mL leupeptin in phosphate-buffered saline solution, pH 7.2 (Bachem California Inc); 1 mg/mL aprotinin (Sigma, St Louis, Mo) and 0.05% sodium azide (Sigma); and 0.5% Triton X-100 (Fisher Scientific International Inc, Pittsburgh, Pa). Samples were homogenized using PowerGen 125 (Fisher Scientific

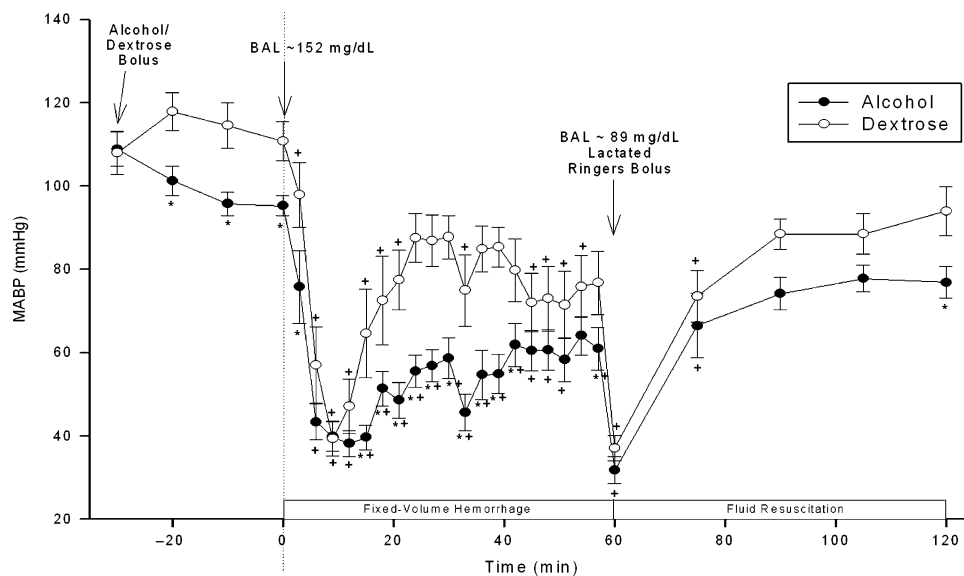


FIG 1. Hemodynamic response to fixed-volume hemorrhagic shock in a rodent model of moderate alcohol intoxication. MABP as a function of time in dextrose-treated ($n = 8$, open circles) and alcohol-treated ($n = 10$, filled circles) animals. Values are means \pm SEM. Asterisk (*) indicates significant difference ($P < 0.05$) from time-matched dextrose-treated animals; plus sign (+), significant difference ($P < 0.05$) from baseline ($t = 0$) values. Statistics were analyzed using 2-way repeated measures ANOVA and showed a difference in mean values among treatment (alcohol or dextrose) and time (both $P < 0.001$), and also an interaction between treatment and time ($P = 0.030$).

International Inc) at the highest speed for up to 1 min and freeze-thawed for 1 cycle. The homogenates were then sonicated (Branson; Branson Ultrasonics Corporation, Danbury, Conn) for 10 min and incubated for 1 hour at 4°C. Tissue homogenates were centrifuged at 44,000 rpm (Beckman Ultracentrifuge; Beckman Coulter, Inc, Fullerton, Calif) for 25 min at 5°C. Tissue cytokine content was measured in the supernatant. Cytokine data are expressed per milligram of tissue protein. Tissue protein content was determined by a method described by Lowry et al. (12) Protein concentration in the sample is proportional to the optical density (at 562 nm) and was calculated using a standard curve generated with bovine serum albumin, ranging from 20 to 2000 $\mu\text{g/mL}$.

Statistical analysis

All data are presented as mean \pm SEM, with the number of animals per group indicated. Statistical analysis of MABP and catecholamines was accomplished by 2-way analysis of variance (ANOVA) with repeated measures. Two-way ANOVA was also used to statistically compare cytokine levels among treatment groups, and *t* tests were used to compare cytokine difference of the means. All pairwise multiple comparisons were done with the Holm-Sidak method. Statistical significance was set at $P < 0.05$.

RESULTS

Blood alcohol concentrations

Intragastric administration of alcohol (1.75 g/kg) resulted in BAC of 152 ± 10 mg/dL at the start of hemorrhage (30 min after alcohol administration). BAC in both sham and hemorrhaged animals decreased significantly from baseline within a 90-min period (corresponding to the 30-min prehemorrhage and the 60-min hemorrhage periods) to 84 ± 20 and 89 ± 11 mg/dL, respectively.

Mean arterial blood pressure

Alcohol administration produced a $14\% \pm 4\%$ decrease in MABP within 30 min to an average of 95 ± 10 mmHg (Fig. 1). These values were $14\% \pm 2\%$ lower than time-matched dextrose-treated animals (115 ± 5 mmHg; $P = 0.051$). An average of $50.3\% \pm 0.6\%$ of the total blood volume per kilogram of body weight was removed from dextrose-treated animals during the 60-min hemorrhagic shock period producing a significant decrease in MABP to 65 ± 10 mmHg at 15 min. Thereafter, MABP recovered to an averaged of 74 ± 8 mmHg during the last 15 min of hemorrhage in dextrose-treated hemorrhaged animals. A similar blood volume was removed from alcohol-treated animals ($49.9\% \pm 0.2\%$ of their total blood volume), producing a marked decrease in MABP to 40 ± 3 mmHg. In contrast with dextrose-treated animals, MABP remained lower for a longer period and averaged 61 ± 5 mmHg during the last 15 min of hemorrhage. Fluid resuscitation restored MABP to prehemorrhagic shock levels (94 ± 6 mmHg) in dextrose-treated animals. Alcohol-treated animals had a similar increase in MABP in response to the initial bolus of Ringer lactate when compared with dextrose-treated controls. However, MABP in alcohol-hemorrhaged animals (77 ± 4 mmHg) was significantly lower than that of time-matched dextrose-hemorrhaged animals at the end of the fluid resuscitation period ($P = 0.031$).

Circulating catecholamines

Baseline epinephrine levels averaged 265 ± 34 pg/mL in the dextrose-treated animals and were not significantly altered by alcohol administration (324 ± 34 pg/mL; Fig. 2). Epinephrine levels in sham animals were not altered from baseline levels throughout the experimental period in either the dextrose-

treated (333 ± 104 pg/mL) or alcohol-treated animals (292 ± 63 pg/mL). Hemorrhage produced a significant increase in epinephrine in both groups [$132\% \pm 89\%$ in dextrose-treated animals ($P = 0.074$); $87\% \pm 28\%$ in alcohol-treated animals ($P = 0.019$)].

Baseline norepinephrine levels averaged 214 ± 26 pg/mL in the dextrose-treated animals and were not altered significantly by alcohol administration (279 ± 22 pg/mL; Fig. 2). Norepinephrine levels in sham animals were not altered from baseline levels throughout the experimental period in either dextrose-treated animals (303 ± 47 pg/mL) or alcohol-treated

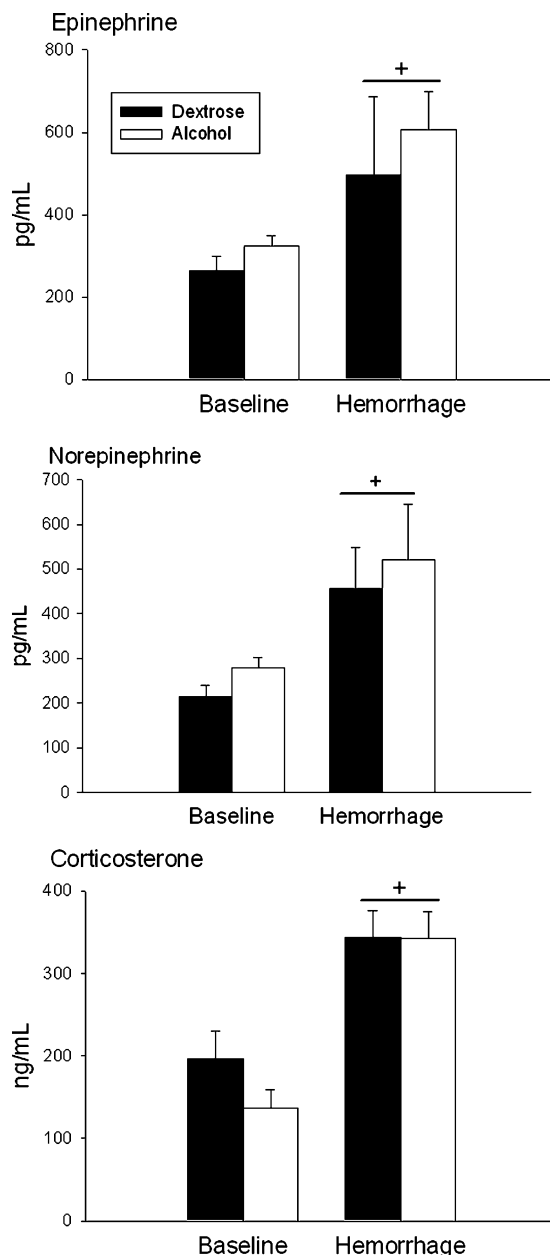


FIG 2. Neuroendocrine response to fixed-volume hemorrhagic shock in a rodent model of moderate alcohol intoxication. Circulating catecholamine (pg/mL) and corticosterone (ng/mL) levels in alcohol-treated and dextrose-treated animals before and at completion of 60-min hemorrhage. Values are means \pm SEM. Plus sign (+) indicates significant difference ($P < 0.05$) from baseline; $n = 5$ to 10 per group. Data was analyzed using 2-way repeated measures ANOVA. No alcohol-hemorrhage interaction was detected.

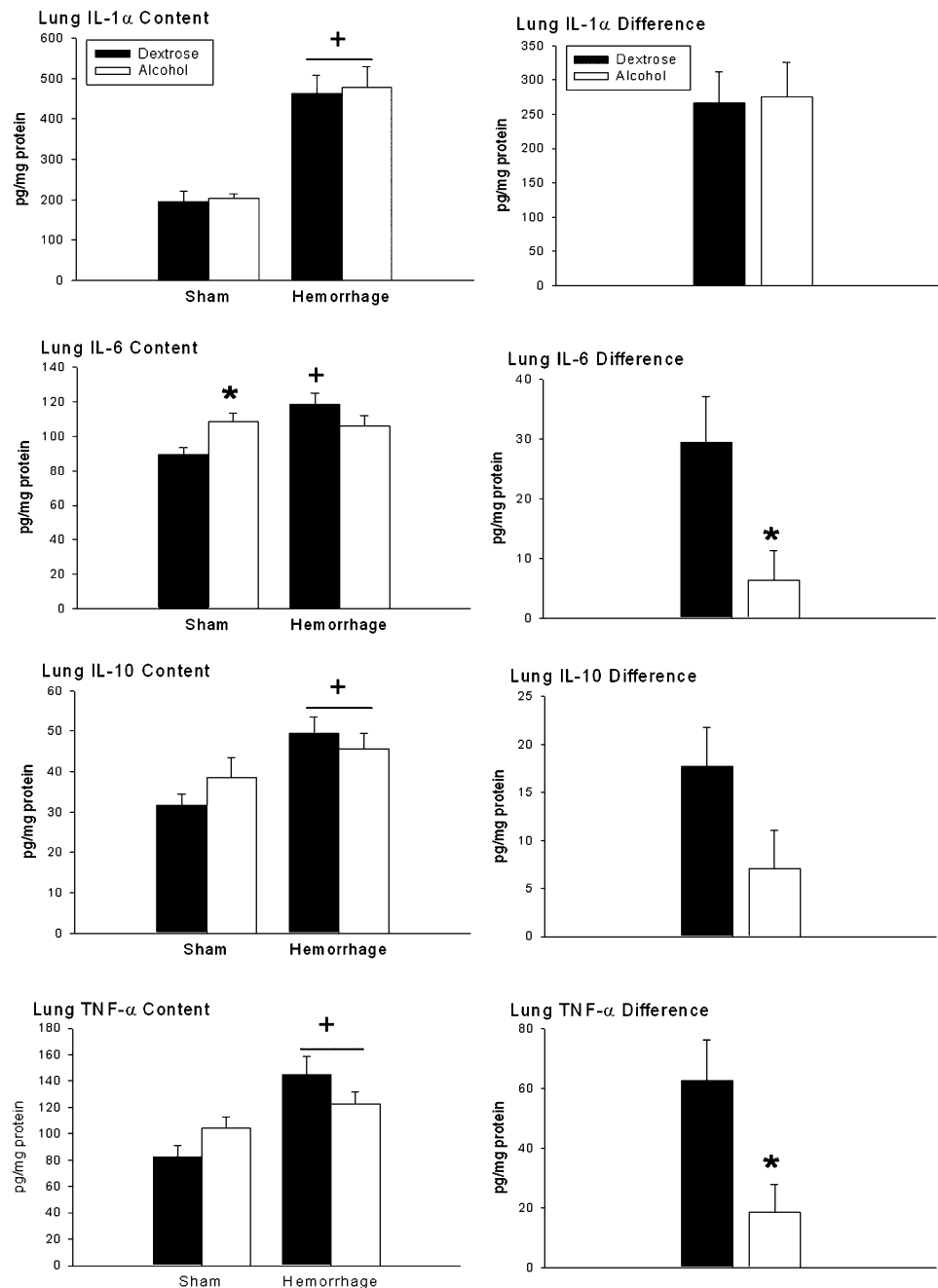


FIG 3. Lung cytokine response to fixed-volume hemorrhagic shock in rodent model of moderate alcohol intoxication. Lung IL-1 α , IL-6, IL-10, and TNF- α content in lung homogenates (pg/mg of protein). Left, Raw data of all experimental groups. Right, Expressed as difference in cytokine expression in response to hemorrhage in dextrose- and alcohol-treated animals. Values are means \pm SEM; $n = 5$ to 10 animals per group. Asterisk (*) indicates significant difference ($P < 0.05$) from time-matched dextrose-treated animals; plus sign (+), significant difference ($P < 0.05$) from corresponding sham. Data was analyzed using 2-way ANOVA followed by Holm-Sidak.

animals (276 ± 35 pg/mL). Hemorrhage produced a significant $13\% \pm 43\%$ increase in norepinephrine in the dextrose-treated animals to an average of 456 ± 92 pg/mL ($P = 0.028$) and a significant $87\% \pm 48\%$ increase in alcohol-treated hemorrhaged animals.

Plasma corticosterone

Baseline corticosterone levels averaged 196 ± 34 ng/mL in the dextrose-treated animals and 136 ± 23 ng/mL in alcohol-treated animals (Fig. 2). Corticosterone levels in sham animals were not altered from baseline levels throughout the experimental period in either dextrose-treated (148 ± 61 ng/mL) or alcohol-treated

(121 ± 41 ng/mL) animals. Hemorrhage produced a significant $75\% \pm 22\%$ increase in corticosterone in the dextrose-treated animals to an average of 344 ± 32 ng/mL, and a $152\% \pm 23\%$ increase in alcohol-treated hemorrhaged animals.

Tissue cytokine content

Lung cytokine responses to hemorrhage and the effects of alcohol are shown in Figure 3. Acute alcohol intoxication increased the levels of lung IL-6 ($22\% \pm 6\%$; $P = 0.020$). This was the only alteration in tissue cytokine expression produced by alcohol administration alone. Hemorrhage resulted in a significant increase in lung IL-1 ($136\% \pm 23\%$; $P = 0.000$),

IL-6 ($33\% \pm 9\%$; $P = 0.001$), IL-10 ($53\% \pm 13\%$; $P = 0.004$), and TNF- α ($76\% \pm 17\%$; $P = 0.001$) expressions. Acute alcohol intoxication prevented the hemorrhage-induced rise in lung IL-6 and TNF- α , but did not alter the lung IL-1 and IL-10 responses.

Alcohol alone had no significant effects on spleen cytokine levels (Fig. 4). Hemorrhage resulted in a significant upregulation of IL-1 ($428\% \pm 85\%$; $P = 0.000$), IL-10 ($177\% \pm 46\%$; $P = 0.001$), and TNF ($92\% \pm 16\%$; $P = 0.003$) expressions in the spleen. Acute alcohol intoxication did not alter spleen IL-1, IL-6, IL-10, or TNF responses.

Myeloperoxidase activity

Alcohol alone had no significant effects on lung MPO activity (Fig. 5). Lung MPO activity was increased by 120% in dextrose-treated animals after hemorrhage and fluid resuscitation ($P = 0.000$). Alcohol did not alter the hemorrhage-induced increase in lung MPO activity.

DISCUSSION

The results from the present study show that acute moderate alcohol intoxication is sufficient to alter hemodynamic and

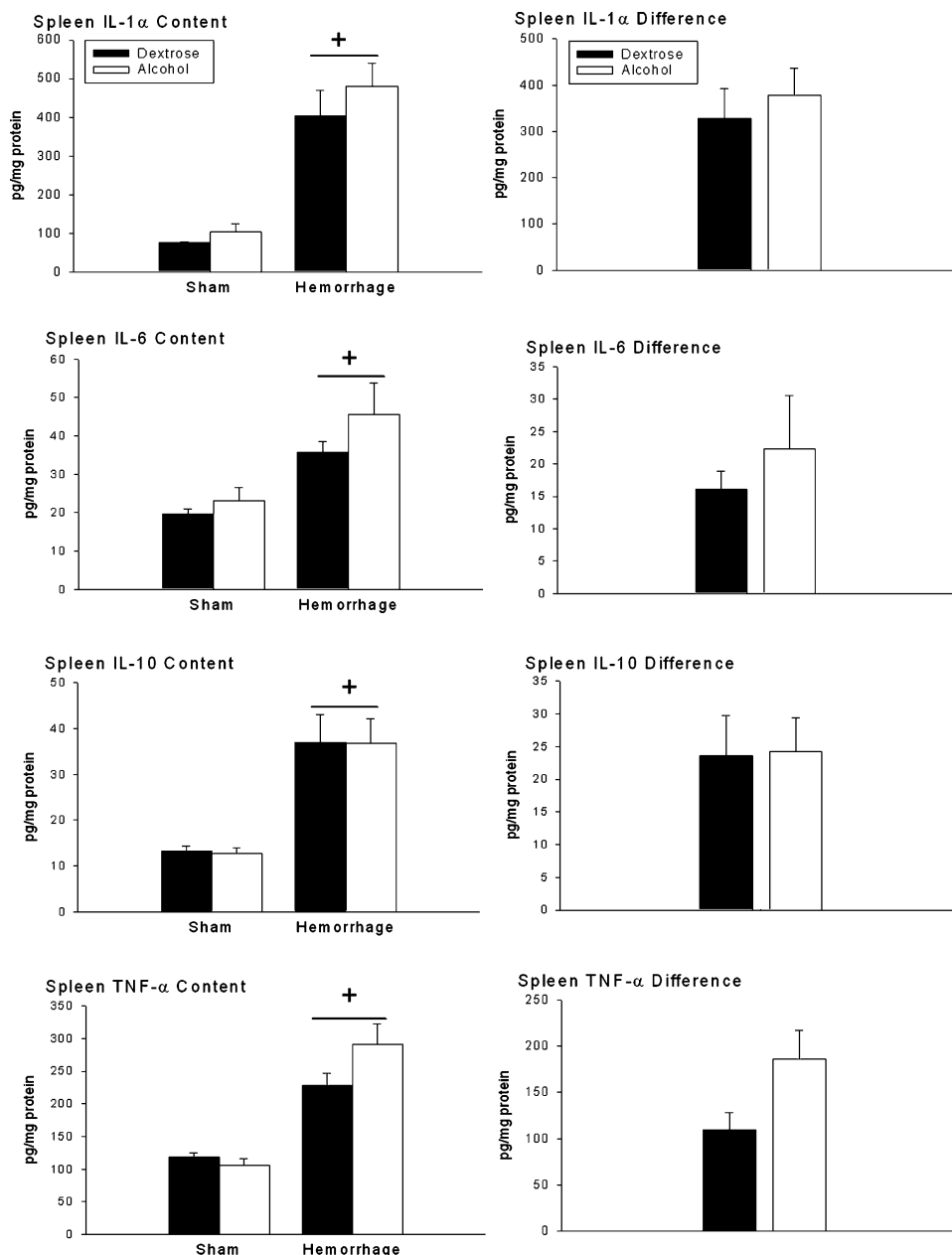


FIG 4. **Spleen cytokine response to fixed-volume hemorrhagic shock in rodent model of moderate alcohol intoxication.** Spleen IL-1 α , IL-6, IL-10, and TNF- α content in lung homogenates (pg/mg of protein). Left, Raw data of all experimental groups. Right, Expressed as difference in cytokine expression in response to hemorrhage in dextrose- and alcohol-treated animals. Values are means \pm SEM; $n = 5$ to 10 animals per group. Asterisk (*) indicates significant difference ($P < 0.05$) from time-matched dextrose-treated animals; plus sign (+), significant difference ($P < 0.05$) from corresponding sham. Data was analyzed using 2-way ANOVA followed by Holm-Sidak.

neuroendocrine counter-regulatory responses to hemorrhagic shock. Our results show that alcohol decreased basal MABP and accentuated the hypotensive response to blood loss, without eliciting a greater compensatory rise in norepinephrine levels. In contrast with the previously reported proinflammatory effects of prolonged alcohol intoxication, acute moderate alcohol intoxication blunted the hemorrhage-induced increases in lung IL-6 and TNF- α expressions.

The greater hypotensive response to blood loss could be reflective of the initial decrease in basal MABP produced by alcohol administration alone, similar to that demonstrated in clinical studies (13). Alternatively, several mechanisms could have contributed to the greater hypotensive response of alcohol-treated animals, including alcohol-induced vasodilation (14), diuresis (15, 16), decreased cardiac output (17), impaired vasoreactivity (18), and depressed myocardial contractility (5, 19–21). Hemorrhage-induced hypotension has been identified as one of the most critical determinants of morbidity and mortality after traumatic injury (22). Thus, one can speculate that alcohol intoxication during hemorrhagic

shock is likely to be a critical negative factor affecting the outcome from hemorrhage.

The results from this study showed a more moderate activation of the neuroendocrine system than that previously reported after fixed-pressure hemorrhagic shock (23–25). It is important to note that, in addition to the different hemorrhagic shock model, the duration of the hypotensive period and the total blood volume removed (approximately 55% vs. 50%) were of lower magnitude in the present study, thus explaining the attenuated epinephrine response observed in this study and, possibly, the absence of alcohol effects in this response. Interestingly, circulating levels of norepinephrine were similar in both groups at the completion of the hemorrhage period, despite the fact that alcohol-treated animals had significantly lower MABP at this time, indicating attenuated sympathetic responsiveness in alcohol-treated animals. This is the subject of further investigation by our laboratory.

In agreement with previous reports from our laboratory, hemorrhage produced an early induction of lung and spleen cytokine (IL-1 α , IL-6, IL-10, and TNF- α) expressions (9). Acute moderate alcohol intoxication blunted the magnitude of the hemorrhage-induced increases in lung IL-6 and TNF- α without affecting spleen responses. Alcohol effects on immune responses have been shown to vary depending on the duration of alcohol exposure, with acute alcohol resulting in most cases in suppression of proinflammatory responses (25–28) and chronic alcohol exposure favoring a proinflammatory response (29–33). Whether the observed alcohol-induced modulation of lung proinflammatory cytokine expression has any significant impact on host defense function is unclear from the present studies and requires further investigation.

Several studies have provided evidence that the early tissue proinflammatory response is tightly regulated by multiple endogenous mechanisms, including neuroendocrine factors (34–37). Glucocorticoids, in particular, have been demonstrated to produce suppression of proinflammatory responses. However, the results from the present study suggest that the alcohol-induced blunting of the hemorrhage-induced cytokine response was not glucocorticoid-mediated. Alcohol administration produced a modest decrease in circulating corticosterone levels and did not alter the hemorrhage-induced increase in corticosterone levels. These findings are in contrast with previous reports in the literature demonstrating that acute intraperitoneal alcohol administration (1.5 g/kg) activates the hypothalamic-pituitary-adrenal axis, resulting in increased circulating levels of corticosterone in rats (38). Among the possible reasons for the different response observed in the present study is the dose or route of alcohol administration.

Hemorrhagic shock resulted in a significant increase in MPO activity, reflecting increased neutrophil infiltration and activation. Alcohol administration did not alter the hemorrhage-induced increase in MPO activity. Although this is consistent with previous observations in our laboratory (25), different effects have been reported with other challenges. Whereas alcohol has been shown to enhance lung MPO responses to burn injury (30), others have shown suppression in neutrophil recruitment in response to an intratracheal challenge with *Streptococcus pneumoniae* (39). The differential response

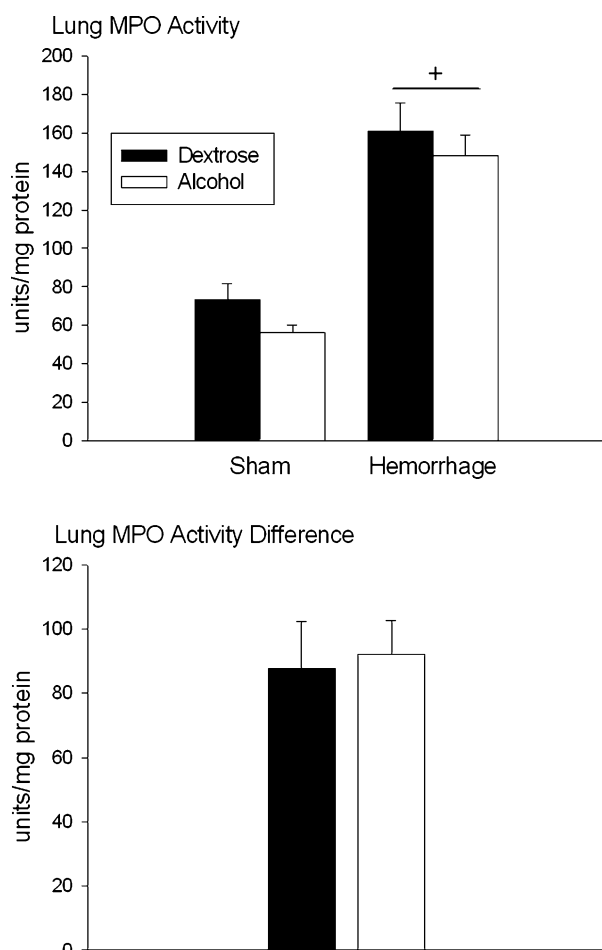


Fig 5. Top, Lung MPO activity in rodent model of moderate alcohol intoxication and fixed-volume hemorrhagic shock lung MPO activity expressed as units per milligram protein. Bottom, Difference in MPO activity in response to hemorrhage in dextrose- and alcohol-treated animals. Values are expressed as means \pm SEM. Plus sign (+) indicates significant difference ($P < 0.05$) from time-matched dextrose-treated animals. Data was analyzed using 2-way ANOVA. No alcohol-hemorrhage interaction was detected.

could be caused by either the duration of alcohol exposure or the specificity of the challenge. Further studies are needed to examine the mechanisms responsible for this differential response.

In conclusion, acute moderate alcohol intoxication impairs the hemodynamic counter-regulatory mechanisms involved in restoring MABP after hemorrhagic shock and fluid resuscitation. Taken together, the results from this and/or previous studies indicate that hemorrhage-induced tissue cytokine expression is differentially affected, depending on the dose of alcohol administered, BAC achieved, duration of alcohol intoxication, and the model of hemorrhagic shock used, underscoring the importance of these variables in formulating conclusions on the impact of alcohol on the outcome from traumatic injury.

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Alcohol Binge Before Trauma/Hemorrhage Impairs Integrity of Host Defense Mechanisms During Recovery

Patrick Greiffenstein, Keisa W. Mathis, Curtis Vande Stouwe, and Patricia E. Molina

Background: Alcohol abuse, both chronic and acute, is a known modulator of immune function and is associated with increased incidence of traumatic injury. Previously, we demonstrated that acute alcohol intoxication before hemorrhagic shock impairs hemodynamic and neuroendocrine counter-regulation, suppresses early lung proinflammatory cytokine expression, and increases mortality from infection during recovery. In the present study, we examined the impact of a 3-day alcohol binge on host responses during trauma/hemorrhage (T×Hem) and following overnight recovery.

Methods: Chronically catheterized, adult male Sprague–Dawley rats were administered an intragastric bolus of alcohol (5 g/kg; 30% w/v) or isocaloric dextrose solution for 3 consecutive days, followed by a 2.5 g/kg dose on day 4 before undergoing full-thickness muscle-crush and fixed pressure (~40 mmHg) hemorrhage and fluid resuscitation (2.4×total blood volume removed).

Results: Alcohol-binge produced a 16% decrease in basal mean arterial blood pressure (MABP), reduced the total blood loss required to reach and to sustain MABP of 40 mmHg, markedly blunted the increase in circulating epinephrine and norepinephrine (20-fold and 3-fold, respectively) levels, and increased immediate mortality from T×Hem. Consistent with our previous reports, significant up-regulation in lung and spleen tumor necrosis factor (TNF)- α and interleukin (IL)-1 α expression was observed immediately following T×Hem and fluid resuscitation. Only the T×Hem-induced increase in lung TNF- α was prevented by binge alcohol administration. Following overnight recovery, significant lipopolysaccharide (LPS)-stimulated release of TNF- α , IL-1 α , IL-6, and IL-10 was observed in cells isolated from blood and the alveolar and pleural compartments from all experimental groups. While T×Hem did not prevent LPS-induced release of TNF- α , IL-1 α , IL-6, or IL-10 at 6 or 24 hours, alcohol binge suppressed TNF- α , IL-1 and IL-6 release, without altering IL-10 response in cells isolated from blood and pleural compartment. No significant modulation of alveolar macrophage response was observed following alcohol binge and T×Hem.

Conclusions: These results indicate that a 3-day alcohol binge results in hemodynamic instability associated with attenuated neuroendocrine activation and increased mortality during T×Hem as well as sustained suppression of the proinflammatory cytokine response of blood and pleural-derived cells to a “second-hit” inflammatory challenge. As a result, we speculate that the net shift toward an anti-inflammatory state may contribute to enhanced susceptibility to infection during the recovery period.

Key Words: Alcohol, Binge, Hemodynamic, Traumatic Injury, Neuroendocrine, Inflammatory, LPS.

CHRONIC AND ACUTE alcohol abuse contributes to a significant number of traumatic injury-related deaths resulting from motor vehicle accidents, penetrating injuries, and burns (Li et al., 1997; Rehm et al., 2003). In addition, alcohol abuse is also associated with a significant number (~50%) of nonlethal traumatic injury-related visits to emergency rooms (Hadfield et al., 2001; Madan et al., 1999; Reyna et al., 1984; Rivara et al., 1993). These alcohol-intoxicated injury victims have greater

risks of mortality, a greater need for intensive care, and are more likely to develop permanent disabilities and acute medical complications such as pneumonia, sepsis, and multiple organ failure (Jurkovich et al., 1993; von Heyman et al., 2002). The greater incidence of complications has been identified to be multifactorial, related not only to injury severity, but to the genetic makeup of the individual, life-time history of alcohol use and abuse (chronic abuse vs acute intoxication), the total amount and duration of alcohol exposure before the traumatic event, the concentration of alcohol present in the blood as well as the need for subsequent interventions that may contribute to the increased morbidity and mortality in this patient population (Jones et al., 1991; Madan et al., 1999; Schwacha et al., 2005; Spies et al., 1996).

Previous studies from our laboratory have shown that the hemodynamic, neuroendocrine, and immune responses to fixed-pressure [mean arterial blood pressure

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(MABP) ~ 40 mmHg] hemorrhagic shock are markedly impaired following a prolonged (15-hour) period of alcohol exposure achieving blood alcohol levels of 190 ± 21 mg/dL (Phelan et al., 2002). Furthermore, we have also shown that the course of hemorrhage and fluid resuscitation is characterized by suppressed polymorphonuclear phagocytic and oxidative burst capacity in alcohol-intoxicated rodents (Molina et al., 2004a) that appeared to be normalized following an overnight recovery period. However, although these studies did not examine systematically the integrity of host defense mechanisms, in subsequent studies using the same model we demonstrated greater morbidity and mortality from bacterial infection during recovery from hemorrhagic shock in alcohol-intoxicated animals (Zambell et al., 2004). Nevertheless, the specific host defense mechanisms that remain impaired during the initial recovery period from hemorrhage in alcohol-intoxicated animals had not been examined previously.

Previous human and animal studies have shown an acute inhibitory effect of alcohol on proinflammatory cytokine production and release in tissues as well as isolated macrophages (Boe et al., 2003; Goral and Kovacs, 2005; Szabo, 1998, 1999; Verma et al., 1993). These immunosuppressive effects of alcohol have been demonstrated shortly following the exposure to either *in vivo* or *in vitro* alcohol, with few studies investigating the combined impact of alcohol and traumatic injury (Choudhry et al., 2000; Faunce et al., 1998; Molina et al., 2004a; Messingham et al., 2002). Moreover, the effects of binge alcohol exposure alone or in combination with traumatic injury and hemorrhagic shock on the integrity of the systemic counterregulatory mechanisms involved in restoring homeostasis during trauma/hemorrhage (T \times Hem) and the subsequent recovery period have not been studied previously. Studies have shown that cellular compartments respond differently to various conditions and stimuli (Deitch et al., 1990). Therefore, analysis of several cellular compartments is necessary to understand more fully the impact of alcohol as well as that of traumatic injury on the immune status of the host. Binge drinking is a frequent pattern of alcohol abuse (SAMHSA, 2005) and it is associated with a higher risk for injury in an otherwise healthy population (Gmel et al., 2006; O'Brien et al., 2006; Savola et al., 2005). Our previous studies have examined the impact of a single alcohol bolus as well as that of a prolonged continuous alcohol administration on outcome from traumatic injury. However, the impact of an alcohol binge pattern, a potentially more relevant model of alcohol abuse, on outcome from T \times Hem has not been investigated previously. Thus, the aim of the present study was to examine the effects of acute intoxication following a 3-day alcohol binge on the immediate tissue cytokine response to T \times Hem and subsequently on the post-injury, compartment-specific immune responsiveness of isolated rat mononuclear cells.

MATERIALS AND METHODS

Animal Preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University Health Sciences Center (LSUHSC) and were in accordance with National Institute of Health guidelines. Specific pathogen-free adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 250 to 300 g were housed in the Division of Animal Care at LSUHSC for at least 1 week before experimental procedures to acclimate them to their surroundings. The animals were fed standard rat chow (Purina, Richmond, IN) and housed in a temperature-controlled environment with 12 hours light/dark cycle exposure. Two to three days before initiating alcohol administration, animals were anesthetized with ketamine/xylazine (90 and 9 mg/kg, respectively) and implanted with sterile vascular (carotid and jugular) and gastric catheters using aseptic surgical procedures as described previously by our laboratory (Phelan et al., 2002). The catheters were routed subcutaneously and exteriorized at the nape of the neck through a 1-cm incision. After surgery, animals were housed in individual cages and allowed to recover completely from anesthesia before providing them with food and water *ad libitum*.

Binge Alcohol Administration

Ethyl alcohol (5 g/kg; 30% w/v) was administered daily as a bolus through the intragastric catheter for 3 consecutive days. Time-matched isocaloric controls received an intragastric bolus of dextrose (8.7 g/kg; 52% w/v). This protocol of alcohol administration was selected as it resembles the route of alcohol intake in humans, the degree of alcohol intoxication achieved during binge-drinking episodes, and results in blood alcohol levels in the range of those reported in trauma patients in emergency rooms across the United States (Maull, 1982; Rivara et al., 1993). A group of animals was used to examine the time course of blood alcohol levels achieved with this protocol (Fig. 1). Animals were lethargic and displayed poor balance and motor coordination during the initial 2 to 3 hours following the daily 5 g/kg dose of alcohol. On the fourth day, animals were administered half the daily alcohol dose (2.5 g/kg) or dextrose (4.35 g/kg) as an intragastric bolus 30 minutes prior T \times Hem. This dose of alcohol did not produce marked lethargy in

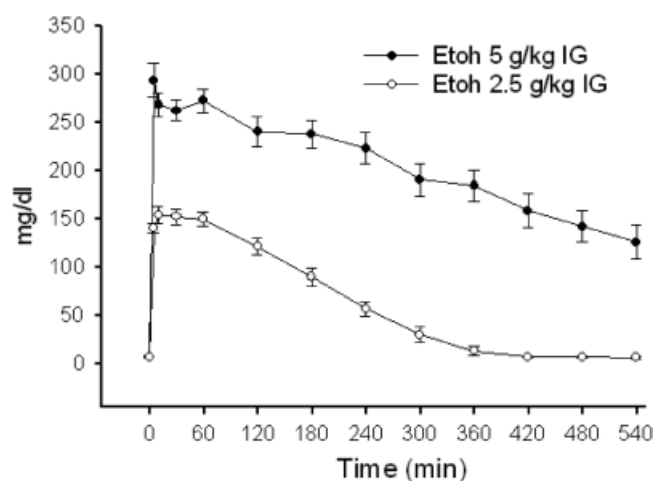


Fig. 1. Blood alcohol concentrations as a function of time following intragastric administration of alcohol (5 and 2.5 g/kg). Animals received daily 5 g/kg alcohol for 3 consecutive days and 1 final bolus (2.5 g/kg) on day 4, 30 min before initiating trauma-hemorrhage. Values are means \pm standard error of the mean, $N = 20$ to 22/group.

the animals but resulted in intoxicating levels of alcohol (165 ± 7 mg/dL) during the T×Hem period.

Trauma, Hemorrhagic Shock, and Resuscitation

All experimental procedures were conducted in conscious and unrestrained animals and were initiated at approximately 7:30 AM. The carotid artery catheter was connected via a pressure transducer to an amplifier that relayed the signals to a data acquisition system (Powerlab, AD Instruments, Colorado Springs, CO). Animals were randomly divided into 4 groups: alcohol-treated no T×Hem (alcohol/sham), dextrose-treated no T×Hem (Dex/sham), alcohol-treated trauma/hemorrhaged (alcohol/T×Hem), and dextrose-treated trauma/hemorrhaged (Dex/T×Hem). Thirty minutes after the administration of the fourth alcohol or dextrose dose, animals were subjected to soft tissue trauma. Briefly, animals were anesthetized by Brevital (20 mg/200 μ L, i.v.), followed by full-thickness gastrocnemius muscle crush with modified pliers (60.85 PSI for a 3-minute duration) as described previously (Molina et al., 2004b). Time-matched sham animals were also anesthetized intravenously with Brevital. Once the animals were fully recovered from Brevital, they were subjected to a fixed-pressure (~ 40 mmHg) hemorrhagic shock for 60 minutes. The aim was to produce similar degrees of hypotension in both dextrose-treated and alcohol-treated animals and to record the differences in percentage of total blood volume removed from each group to achieve target blood pressure. The mean arterial blood pressure was monitored throughout the duration of the experiment and recorded and averaged every 3 minutes during the hemorrhage period and blood removal was adjusted accordingly. Blood pressure was monitored every 15 minutes throughout the fluid resuscitation period. Animals were not heparinized before, during, or after hemorrhage. At the end of the 60-minute hemorrhage, the animals received an intravenous bolus of warmed (34°C) Ringer's lactate to provide 40% of the total blood volume removed, followed by a constant intravenous infusion over a 60-minute period of Ringer's lactate equal to 2 times the total volume of blood withdrawn. In total, 2.4 times the blood volume removed was replaced with Ringer's lactate. Two sets of experiments were performed. In the first study, the hemodynamic, neuroendocrine, and tissue cytokine responses were examined. For this purpose, animals were killed at the end of the fluid resuscitation period ($T = 120$ minutes) by an intravenous injection of sodium pentobarbital (125 mg/kg), followed by exsanguinations. Tissues (lung and spleen) were excised immediately, washed in normal saline, freeze-clamped with liquid nitrogen, and stored at -80°C . In the second study, we examined the ex vivo responsiveness of mononuclear cells to a "second-hit" inflammatory challenge. For this study, animals were returned to their cages at the completion of T×Hem and fluid resuscitation, provided access to food and water ad libitum, and allowed to recover overnight (18 hours). Mortality from trauma hemorrhage and fluid resuscitation and during the initial overnight recovery period was recorded in both sets of studies.

Blood, Alveolar, and Pleural Cell Isolation

The morning after completion of T×Hem and fluid resuscitation, animals were anesthetized with ketamine/xylazine (90 and 9 mg/kg, respectively) before undergoing laparotomy to expose the inferior vena cava. Using a 21-gauge needle attached to a heparinized 10 cc syringe, approximately 8 mL of blood was obtained from the vena cava, transferred to a 15 mL polypropylene tube at room temperature, and immediately centrifuged at $500\times g$ for 15 minutes with low brake. Plasma was collected and frozen at -80°C until further analysis. The pellet was gently resuspended in 15 mL of sterile RPMI-1640 medium containing penicillin–streptomycin–glutamine ($100\times$) solution (Gibco[®], Carlsbad, CA) until sample collection was completed from all animals to process all samples simultaneously.

The suspended pellet was then layered onto 10 mL of Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) in a 50 mL sterile polypropylene tube, which was then centrifuged ($610\times g$ for 30 minutes without brake) for isolation of peripheral blood mononuclear cells (PBMCs) as described previously (Boyum, 1968). Most of the medium above the interface was removed and the interface layer of cells (the mononuclear cell layer) was transferred into a 15 mL polypropylene tube to which 8 mL of plain RPMI-1640 was added and mixed well by gentle inversion. Following a second centrifugation step ($610\times g$ for 15 minutes with low brake), the liquid layer was removed and discarded and the pellets were preserved. The pellets then underwent hypotonic lysis by resuspending in sterile deionized water for 20 seconds, followed by addition of equivalent volumes of $2\times$ phosphate-buffered saline (PBS; Gibco[®]) to achieve isotonicity. The cell pellets were then washed twice more. Each wash entailed centrifugation at $610\times g$ for 5 minutes and resuspension in 5 mL of RPMI complete media (RPMI-1640 with penicillin–streptomycin–glutamine $100\times$ solution as well as 5% heat-inactivated fetal calf sera; Gibco[®]). The final resuspension was also in RPMI complete media.

Immediately following venipuncture, pleural and bronchoalveolar lavage (BAL) were performed. Briefly, an angiocatheter (18-gauge, Becton Dickinson, Sandy, UT) was used to carefully penetrate the pleural space, without puncturing the lung, and 10 mL of chilled (4°C) heparinized (1,000 U/L; heparin sulfate, Baxter Healthcare Corp., Deerfield, IL) sterile PBS was infused into the cavity. After gently massaging the chest to ensure adequate distribution of fluid throughout the cavity, pleural lavage (PLUL) fluid was aspirated and collected (average recovery was 9 mL) into sterile 15 mL polypropylene tubes and kept on ice. The heart–lung block was then removed and BAL was performed. Briefly, the lungs were lavaged with 30 mL cold PBS via an intratracheal tube. The collected BAL fluid was transferred into 50 mL polypropylene tubes and put on ice. Once all BAL and PLUL samples were collected, they were simultaneously centrifuged at $610\times g$ for 5 minutes and the supernatant was discarded. The pelleted cells underwent hypotonic lysis (as described above), followed by resuspension in RPMI complete medium.

Total cell counts for all samples (PBMC, BAL, and PLUL) were obtained using a hemocytometer. Cell viability was $>95\%$, as determined by Trypan blue exclusion and lactate dehydrogenase (LDH) levels, and did not differ between treatment groups (data not shown). Differential cell counts were determined using Cytospin (Thermo Electron Corp., Waltham, MA) and commercially available Diff-Quik[®] Stain Set (Baxter Healthcare Corp., McGaw Park, IL). All procedures were carried out using a strict sterile technique.

Cell Plating and LPS Challenge

Cells (1.25×10^6 PBMC's; 2.5×10^5 pleural-derived and alveolar-derived cells) were plated into 24 wells cell culture cluster flat-bottomed plates (Corning Incorporated, Corning, NY) and challenged with highly purified lipopolysaccharide (LPS) (*Escherichia coli* serotype 0111:B4, List Biological Laboratories Inc., Campbell, CA) in RPMI complete medium for a final LPS concentration of 1 $\mu\text{g/mL}$. Unstimulated controls (RPMI complete media alone) were included in all experimental groups. Cell cultures were incubated at 5% CO_2 in a 37°C air incubator (Sanyo Scientific, Bensenville, IL). Cell culture supernatants were removed at 6 and 24 hours, transferred to a 1.5 mL polypropylene tube, centrifuged ($610\times g$ for 5 minutes at 4°C), and supernatants stored at -80°C until analyzed for cytokine concentrations. We chose 6 and 24 hours based on previous reports in the literature demonstrating that the peak proinflammatory response to LPS challenge occurs within that time frame (Xing and Remick, 2003).

Tissue Preparation

Frozen tissue samples were weighed and placed in a homogenization buffer at 4 °C (100 mg tissue/1 mL of buffer). The homogenization buffer consisted of a protease inhibitor solution containing 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mg/mL pepstatin A, and 1 mg/mL leupeptin in PBS solution (pH 7.2), (all from Bachem, Torrance, CA); 1 mg/mL aprotinin and 0.05% sodium azide, (both from Sigma, St. Louis, MO); and 0.5% Triton X-100 (Fisher Scientific, Pittsburgh, PA). Samples were homogenized using PowerGen 125 (Fisher Scientific) at the highest speed for up to 1 minute and freeze-thawed for 1 cycle. The homogenates were then sonicated (Branson®, Branson Ultrasonic Corporation, Danbury, CT) for 10 minutes and incubated for 1 hour at 4 °C. Tissue homogenates were centrifuged at 44,000 rpm (87,000×g) (Beckman Ultracentrifuge, Fullerton, CA) for 25 minutes at 5 °C. Tissue cytokine content was measured in the supernatant. Cytokine data are expressed per milligram of tissue protein. Tissue protein content was determined by a method described by Lowry et al. (1951). Protein concentration in the sample is proportional to the optical density ($\lambda = 562$ nm) and was calculated using a standard curve generated with bovine serum albumin, ranging from 20 to 2,000 $\mu\text{g/mL}$.

Analytical Procedures

Arterial blood samples were collected in chilled heparinized syringes and aliquots were placed in tubes containing aprotinin (Sigma) at 10 $\mu\text{L/mL}$ of blood, or catecholamine preservative [9% ethylenediaminetetraacetic acid (EDTA), 6% glutathione, and dH_2O at a pH of 6.0–7.4] at 20 $\mu\text{L/mL}$ of blood. Blood samples were centrifuged for 15 minutes at 10,000 rpm (9300×g) for plasma separation. Blood alcohol concentrations were measured using an amperometric oxygen electrode (Analox Instruments Limited, London, U.K.). Creatinine kinase concentrations in plasma were determined as an index of muscle injury using a commercially available enzymatic assay according to the manufacturer's instructions (Stanbio Laboratory, Boerne, TX). High-performance liquid chromatography (HPLC) was used to quantify circulating epinephrine and norepinephrine levels. Plasma samples were spiked with 30 μL of 3,4-dihydroxybenzylamine (DHBA), the internal control, and absorbed into a small quantity of aluminum oxide (alumina). The samples were quantified for circulating epinephrine and norepinephrine levels using a HPLC system consisting of a chromatographic analyzer with a catecholamine column and an electrochemical detector (Bioanalytic Systems, West Lafayette, IN). Corticosterone levels were measured as an index of activation of the hypothalamic–pituitary–adrenal axis, a hallmark of the stress response, using a commercially available rat-specific radioimmunoassay (DPC Diagnostic Products, Los Angeles, CA).

Tissue (lung and spleen) and cell culture supernatant proinflammatory [interleukin (IL)-1 α , IL-6, and tumor necrosis factor (TNF)- α] and anti-inflammatory (IL-10) cytokine concentrations were determined using rat-specific enzyme-linked immunosorbent assays (ELISA) (BioSource, International, Camarillo, CA) according to the manufacturer's instructions and as reported previously by our laboratory (Phelan et al., 2002). Values were calculated as picogram per milligram of protein and expressed and as percent of time-matched controls for tissue samples and as picogram per milliliter for culture supernatants. Myeloperoxidase (MPO) activity was determined as an index of neutrophil infiltration using a modified enzymatic approach (Clark et al., 1975). Briefly, tissues were homogenized in buffer (100 mg/ml of 20 mM phosphate/0.1 mM EDTA, pH 7.4) and homogenates were subjected to centrifugation (10,000×g for 5 minutes) at 4 °C. The supernatant was discarded and the pellets were immediately frozen in liquid nitrogen. Pellets were resuspended in 3 mL buffer (50 mM phosphate buffer/0.5% HTAB, pH 6.0), sonicated for 10 minutes, subjected to 2 cycles of freeze-thaw, and sonicated once more before a final centrifugation

step (20,000×g for 15 minutes) at 4 °C. Myeloperoxidase activity in supernatants was assessed by measuring the change in absorbance at 450 nm (Ultraspec, Pharmacia Biotech, Pittsburgh, PA) in 150 μL reaction buffer (80 mM PBS, pH 5.4, containing 1.5 mM H_2O_2 and 1.6 mM 3,3',5,5'-tetramethylbenzidine (TMB) at room temperature. The reaction was stopped with 50 μL of 3 M H_2SO_4 (pH 3.0). Under these conditions, 1 U of MPO activity produces an increase in absorbance of around 5 per minute resulting from decomposition of H_2O_2 and oxidation of TMB. Myeloperoxidase activity (U/g/min) is expressed as the amount of enzyme necessary to produce a change in the absorbency of 1.0/min/g of tissue.

Statistical Analysis

All data are presented as mean \pm standard error of the mean with the number of animals per group indicated. Statistical analysis of MABP, neuroendocrine mediators, cell counts, and in vitro LPS-induced cytokine release was accomplished by 2-way analysis of variance (ANOVA) with repeated measures. Two-way ANOVA was also used to statistically compare cytokine levels among treatment groups. All pair-wise multiple comparisons were performed with the Holm–Sidak method. Differences in survival rate were analyzed using the Fisher Exact Test. Statistical significance was set at $p < 0.05$.

RESULTS

Blood Alcohol Concentrations During 3-Day Binge and at the Time of T \times Hem (Fig. 1)

Daily intragastric administration of alcohol (5 g/kg) produced peak blood alcohol concentrations of 293 ± 13 mg/dL within 5 minutes of administration. Blood alcohol levels remained above 100 mg/dL for 9 hours. Intragastric administration of alcohol 2.5 g/kg produced peak blood alcohol concentrations (154 ± 9 mg/dL) at 10 minutes and remained above 100 mg/dL for 2 hours after intragastric administration. Thus, on the day of the study, blood alcohol concentrations were within intoxicating levels throughout the duration of the experimental period.

Impact of 3-Day Alcohol Binge on Hemodynamic Counter-regulation to T \times Hem

Soft tissue injury resulted in a significant and sustained (until the end of fluid resuscitation) elevation in circulating levels of creatine kinase from an average of 73 ± 9 U/L in dextrose-treated sham animals to 249 ± 44 U/L. Binge alcohol did not alter the basal (79 ± 10 U/L) or the trauma-induced increase in creatine kinase (270 ± 62 U/L) levels, suggesting comparable tissue injury in both the dextrose and alcohol-treated trauma/hemorrhaged animals.

Alcohol administration produced a significant $16 \pm 3\%$ ($p < 0.001$) decrease in MABP within 30 minutes to an average of 93 ± 3 mmHg (Fig. 2A). These values were $21 \pm \%$ lower than those of time-matched dextrose-treated animals (113 ± 3 mmHg; $p = 0.002$). An average of $65 \pm 2\%$ of the total blood volume per kilogram of body weight was removed from dextrose-treated animals during the 60-minute hemorrhagic shock period to reach an MABP of ~ 40 mmHg. A significantly lower amount of

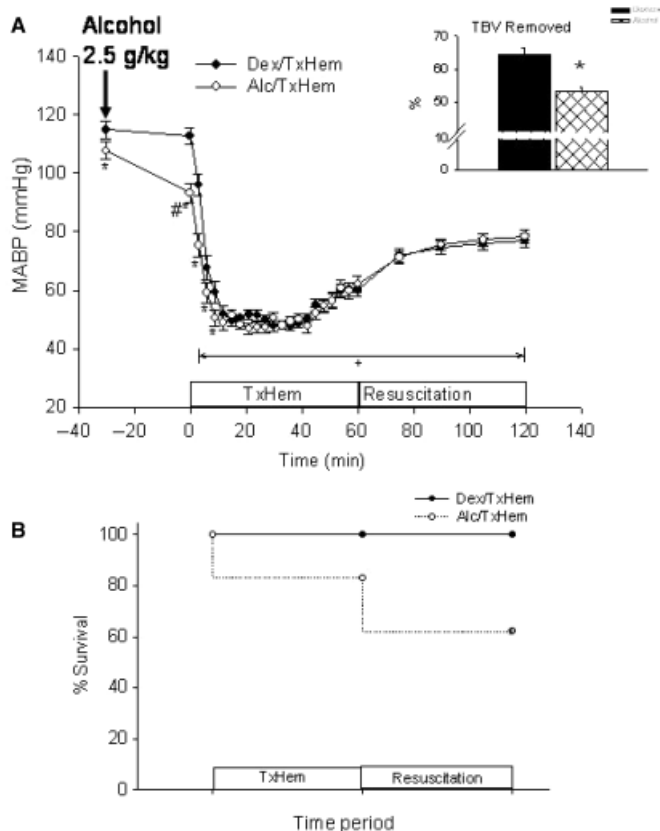


Fig. 2. (A). Mean arterial blood pressure (MABP) as a function of time (min) in dextrose-treated (●) and alcohol-treated animals (○) during trauma and fixed-pressure hemorrhage [trauma/hemorrhage (T×Hem)] and throughout the fluid resuscitation period. A smaller percent of total blood volume (TBV) was removed from alcohol-treated animals (cross-hatched bar) to achieve hypotension similar to that of dextrose-treated animals (solid bar) as shown in the inset. $N = 15$ to 20/group. Values are means \pm standard error of the mean. * $p < 0.05$ versus time-matched dextrose-treated, $^{\dagger}p < 0.05$ versus baseline ($T = 0$) values. **(B)** Percent survival of dextrose-treated (●) and alcohol-treated animals (○). Values are percent (%) estimated at completion of trauma and fixed-pressure hemorrhage (T×Hem) and following completion of the fluid resuscitation period. No additional T×Hem-related mortality was recorded during the overnight recovery period in either group. * $p < 0.05$ versus time-matched dextrose-treated animals.

blood was removed from the alcohol-treated animals to achieve the same target blood pressure ($53 \pm 2\%$ of the total blood volume) as that of the dextrose-treated animals. Alcohol-treated animals had a similar increase in blood pressure in response to the initial bolus of Ringer's lactate to that of the dextrose-treated controls. Fluid resuscitation did not restore blood pressure to basal levels ($T = 0$) in either group of animals. The mean arterial blood pressure in both alcohol and dextrose-treated animals was $\sim 20\%$ lower than baseline values at the completion of the fluid resuscitation period ($p < 0.001$).

Hematocrit averaged $43 \pm 2\%$ during basal in dextrose-treated animals and decreased to $24 \pm 1\%$ at the end of T×Hem and $20 \pm 1\%$ at completion of fluid resuscitation. Alcohol binge did not alter basal hematocrit values ($42 \pm 3\%$) but attenuated the decline in hematocrit fol-

lowing blood loss to an average of $31 \pm 1\%$. Following completion of fluid resuscitation, hematocrit averaged $23 \pm 1\%$ in alcohol-treated trauma/hemorrhaged animals and this was not different from that of dextrose-treated T×Hem animals at that time point.

Survival from T×Hem and fluid resuscitation was 100% in dextrose-treated animals but averaged 80% immediately at the completion of T×Hem. Moreover, while T×Hem did not result in mortality in the dextrose-treated animals, a significantly greater mortality (40%, $p < 0.05$) was observed in alcohol-treated animals at completion of T×Hem and fluid resuscitation (Fig. 2B). No additional mortality was recorded during the overnight recovery period in either the dextrose-treated or alcohol-treated animals.

Effect of Alcohol Binge on Neuroendocrine Response to Hemorrhage

Baseline epinephrine levels averaged 191 ± 20 pg/mL in the dextrose-treated animals and were not significantly altered by alcohol administration (255 ± 31 pg/mL) or by soft tissue injury alone (227 ± 28 pg/mL) (Fig. 3). Trauma/hemorrhage produced a significant 20-fold increase in epinephrine in dextrose-treated animals to an average of $4,140 \pm 876$ pg/mL ($p = 0.001$). Alcohol-binge significantly blunted the T×Hem-induced increase ($436 \pm 92\%$; $p = 0.037$) in circulating epinephrine levels. Epinephrine levels in sham animals were not altered from baseline levels throughout the experimental period in either the dextrose-treated (257 ± 52 pg/mL) or alcohol-treated animals (241 ± 35 pg/mL).

Baseline norepinephrine levels averaged 208 ± 21 pg/mL in the dextrose-treated animals and were not altered significantly by alcohol administration (305 ± 22 pg/mL) or by soft tissue injury alone (196 ± 23 pg/mL) (Fig. 3). Trauma/hemorrhage produced a significant 340 \pm 129% increase in circulating norepinephrine levels in the dextrose-treated animals to an average of $1,157 \pm 339$ pg/mL ($p = 0.001$). Alcohol treatment completely prevented the T×Hem-induced increase in norepinephrine. Norepinephrine levels in sham animals were not altered from baseline levels throughout the experimental period in either dextrose-treated animals (263 ± 5 pg/mL) or alcohol-treated animals (362 ± 52 pg/mL).

Baseline corticosterone levels averaged 216 ± 34 ng/mL in the dextrose-treated animals and increased ($68 \pm 9\%$) significantly ($p = 0.003$) following soft tissue injury alone and at the completion of T×Hem to an average of 470 ± 25 ng/mL. Alcohol binge alone significantly decreased basal corticosterone levels to approximately 50% of basal values of dextrose-treated animals, did not alter the increase in corticosterone levels following soft tissue injury, but did accentuate the magnitude of the T×Hem-induced increase an average of 4-fold (Fig. 3).

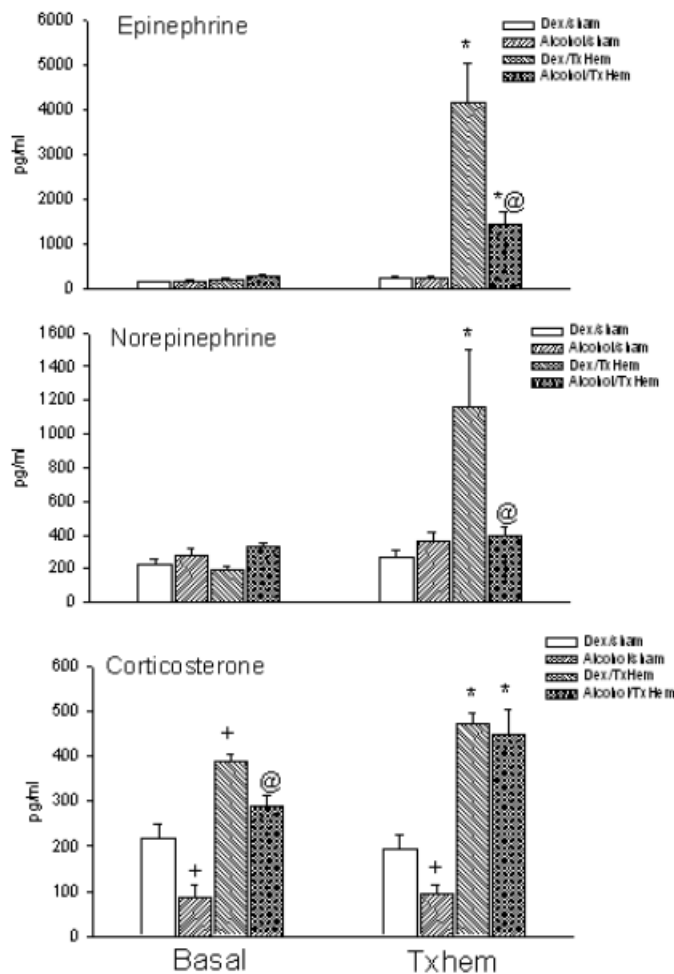


Fig. 3. Circulating epinephrine (pg/mL), norepinephrine (pg/mL), and corticosterone (ng/mL) levels in dextrose-treated sham (open bar), binge-alcohol sham (left-hatched bar), dextrose-treated trauma/hemorrhage (T×Hem) (right-hatched bar), and alcohol-binge T×Hem (cross-hatched bar) before and at completion of 60-min hemorrhage. Values are means \pm standard error of the mean, * $p < 0.05$ versus basal value, * $p < 0.05$ versus dextrose-treated sham, @ $p < 0.05$ versus dextrose-treated trauma/hemorrhaged (Dex/T×Hem). $N = 7$ to 10/group.

Effect of Alcohol Binge on Early Tissue Cytokine Expression and MPO Activity Following T×Hem and Fluid Resuscitation (Fig. 4)

Trauma/hemorrhage resulted in a significant increase in lung TNF- α (40%) and IL-1 (150%) expression. Binge alcohol alone did not alter lung cytokine expression, but did blunt the T×Hem-induced increase in lung TNF- α without altering lung IL-1 response. Trauma/hemorrhage resulted in a significant increase in spleen TNF- α (~50%) and IL-1 (>2-fold) expression. Binge alcohol alone did not alter spleen cytokine expression nor did it alter the T×Hem-induced increase in spleen IL-1 and TNF- α .

Lung MPO activity was significantly ($p = 0.001$) increased (113%) in dextrose-treated animals at completion of T×Hem and fluid resuscitation. Alcohol alone had no significant effects on lung MPO activity, nor did it alter

the T×Hem-induced increase in lung MPO activity (data not shown).

Effect of Alcohol Binge on the Integrity of Cellular Responsiveness to LPS During Recovery From T×Hem

The integrity of LPS-induced cytokine release was examined in cells obtained from 3 different compartments: blood, pleural space, and alveolar compartment.

Peripheral Blood Mononuclear Cells (Fig. 5)

Differential cell counts were similar in all experimental groups and averaged 72% lymphocytes, 26% monocytes, and <2% polymorphonuclear cells. Unstimulated cytokine release was negligible in cell cultures from all experimental groups. In vitro LPS stimulation resulted in a significant increase in TNF- α (6 hours: $2,412 \pm 464$ pg/mL and 24 hours: $2,637 \pm 545$ pg/mL), IL-6 (6 hours: 176 ± 35 pg/mL and 24 hours: 933 ± 70 pg/mL), IL-1 α (24 hours: 363 ± 38 pg/mL), and IL-10 (24 hours: 69 ± 16 pg/mL) release from PBMC isolated from dextrose-treated sham animals. Trauma/hemorrhage did not alter LPS-induced PBMC release of TNF- α , IL-1 α , IL-6, or IL-10 at either 6 or 24 hours. Alcohol binge resulted in a significant 40% suppression of LPS-induced TNF- α release from PBMC's at 24 hours, which was further accentuated in PBMCs isolated from alcohol-treated T×Hem animals. Lipopolysaccharide-induced release of IL-6 increased from 6 to 24 hours of incubation in cells from all experimental groups. Alcohol binge alone did not alter LPS-induced IL-6 release but it significantly suppressed IL-6 release from cells obtained from T×Hem animals at both 6 and 24 hours (70 and 50%, respectively, $p < 0.05$). Lipopolysaccharide-induced IL-1 and IL-10 release was not detectable at 6 hours in any of the experimental groups. Interleukin-1 α release was significantly suppressed (60%, $p < 0.05$) only in cells obtained from alcohol-treated T×Hem animals, while IL-10 release was not different among groups.

Bronchoalveolar Macrophages (Fig. 6)

Differential cell counts averaged >95% macrophages in all experimental groups. Unstimulated cytokine release was negligible in cell cultures from all experimental groups. In vitro LPS stimulation of bronchoalveolar macrophages obtained from dextrose-treated sham animals resulted in a significant increase in TNF- α (6 hours: $5,431 \pm 893$ pg/mL and 24 hours: $5,431 \pm 836$ pg/mL), IL-6 (6 hours: 276 ± 50 pg/mL and 24 hours: 824 ± 128 pg/mL), and IL-1 α (24 hours: 312 ± 69 pg/mL), but did not result in a significant release of IL-10 at 6 hours and a relatively modest IL-10 response (62 ± 14 pg/mL) at 24 hours. In fact, IL-10 release was only detected in 20 to 40% of cell cultures at 24 hours. As observed in PBMCs IL-6 concentrations were higher at 24 hours than at 6 hours for all experimental groups. Lipopolysaccharide-induced alveolar macrophage release of

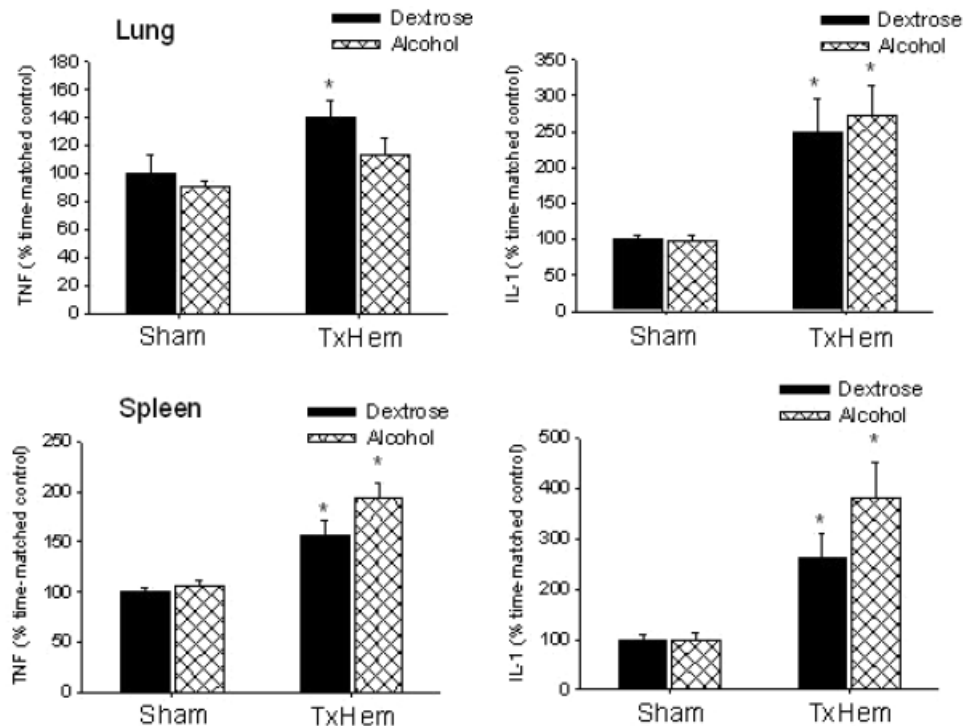


Fig. 4. Lung and spleen expression of tumor necrosis factor (TNF- α) and interleukin 1 (IL-1 α) expressed as percent of time-matched control at the completion of T \times Hem in dextrose-treated (solid bars) and alcohol-treated animals (cross-hatched bars). Values are means \pm standard error of the mean, $N = 5$ to 10 animals/group. * $p < 0.05$ versus time-matched sham animals.

TNF- α , IL-1 α , and IL-6 was not affected by binge alcohol, T \times Hem, or by the combination of binge alcohol and T \times Hem at either 6 or 24 hours.

Pleural Cells (Fig. 7)

Differential cell counts were similar in all experimental groups and averaged 12% lymphocytes, 62% monocytes,

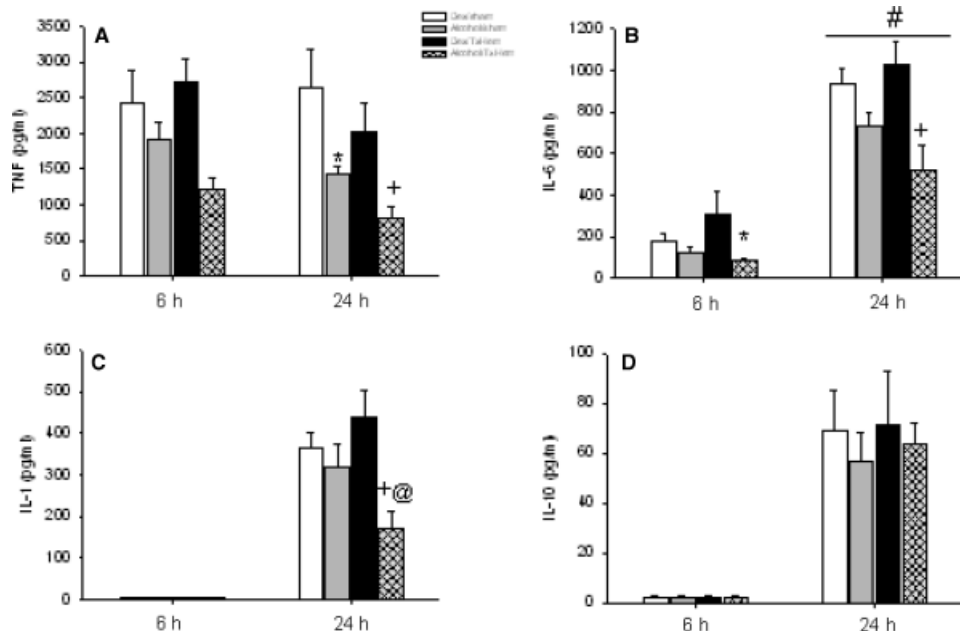


Fig. 5. Concentrations of tumor necrosis factor (TNF)- α (A), interleukin (IL)-6 (B), IL-1 α (C), IL-10 (D) in culture supernatants of lipopolysaccharide-stimulated peripheral blood mononuclear cells isolated from dextrose-treated controls (Dex/sham), alcohol-treated sham (alcohol/sham), dextrose-treated hemorrhage (Dex/T \times Hem), and alcohol-treated hemorrhaged (alcohol/T \times Hem) animals. Values are mean \pm standard error of the mean, $N = 7$ to 10/group determined at 6 and 24 h of incubation. # $p < 0.05$ versus respective 6 h value, * $p < 0.05$ versus time-matched Dex/sham, + $p < 0.05$ versus time-matched Dex/T \times Hem. @ $p < 0.05$ versus time-matched alcohol/sham animals. Interleukin-1 and IL-10 concentrations were not detectable at 6 h.

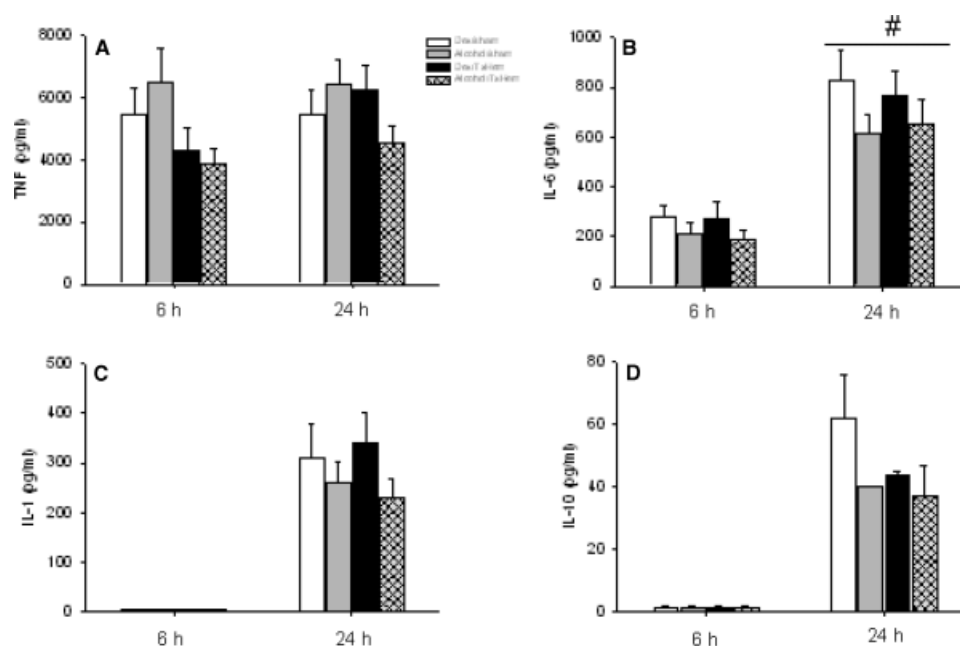


Fig. 6. Concentrations of tumor necrosis factor (TNF)- α (A), interleukin (IL)-6 (B), IL-1 α (C), and IL-10 (D) in culture supernatants of lipopolysaccharide-stimulated alveolar macrophages isolated from dextrose-treated controls (Dex/sham), alcohol-treated sham (alcohol/sham), dextrose-treated hemorrhage (Dex/T \times H), and alcohol-treated hemorrhaged (alcohol/T \times H) animals. Values are mean \pm standard error of the mean, $N = 7$ to 10/group determined at 6 and 24 h of incubation. # $p < 0.05$ versus respective 6-h value. Interleukin-1 α and IL-10 concentrations were not detectable at 6 h.

13% polymorphonuclear, and 13% basophil cells. Unstimulated cytokine release was negligible in cell cultures from all experimental groups. In vitro LPS stimulation resulted in a significant increase in TNF- α (6 hours: 929 ± 133 pg/mL and 24 hours: 843 ± 125 pg/mL), IL-6

(6 hours: $1,354 \pm 199$ pg/mL and 24 hours: $3,490 \pm 446$ pg/mL), IL-1 α (24 hours: 591 ± 43 pg/mL), and IL-10 (24 hours: 66 ± 7 pg/mL) release from pleural cells isolated from dextrose-treated sham animals. Trauma/hemorrhage did not alter LPS-induced pleural cell release of TNF- α ,

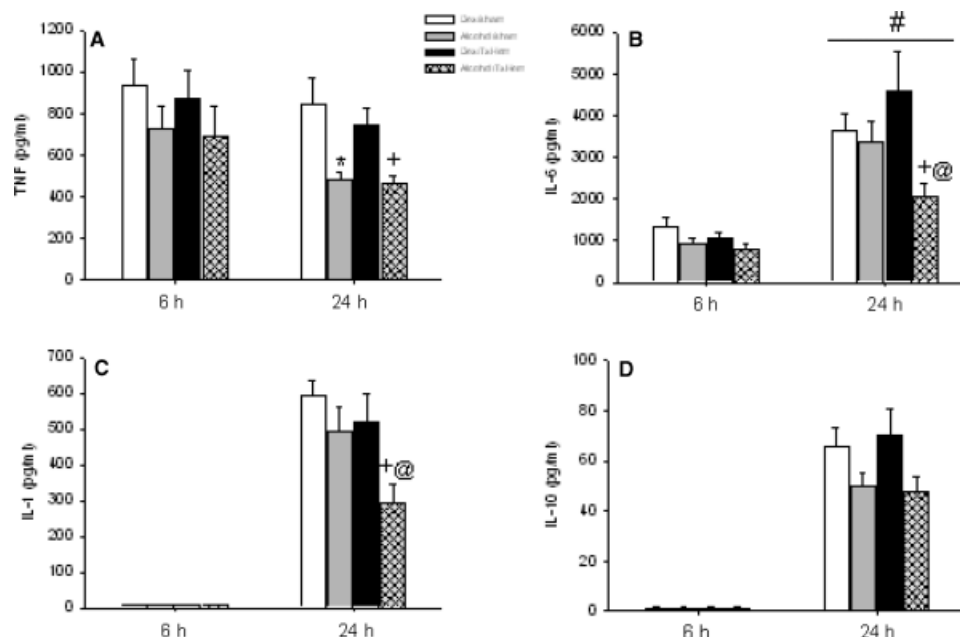


Fig. 7. Concentrations of tumor necrosis factor (TNF)- α (A), interleukin (IL)-6 (B), IL-1 (C), and IL-10 (D) in culture supernatants of lipopolysaccharide-stimulated pleural compartment cells isolated from dextrose-treated controls (Dex/sham), alcohol-treated sham (alcohol/sham), dextrose-treated hemorrhage (Dex/T \times Hem), and alcohol-treated hemorrhaged (alcohol/T \times Hem) animals. Values are mean \pm standard error of the mean, $N = 7$ to 10/group determined at 6 and 24 h of incubation. # $p < 0.05$ versus respective 6-h value, * $p < 0.05$ versus time-matched Dex/sham, + $p < 0.05$ versus time-matched Dex/T \times Hem, @ $p < 0.05$ versus time-matched alcohol/sham animals. Interleukin-1 α and IL-10 concentrations were not detectable at 6 h.

IL-6, IL-1 α , or IL-10 at either 6 or 24 hours. Alcohol binge resulted in a significant 40% suppression of LPS-induced TNF- α release at 24 hours from pleural cells obtained from sham and T \times Hem animals. Supernatant concentrations of IL-6 increased progressively from 6 to 24 hours of incubation in cells obtained from all experimental groups. The 6-hour response was not altered by alcohol binge, T \times Hem, or by the combination of alcohol binge and T \times Hem. However, the 24-hour response was significantly attenuated in cells obtained from alcohol-treated T \times Hem animals. Lipopolysaccharide-induced IL-1 and IL-10 release was not detectable at 6 hours in any of the experimental groups. The LPS-induced increase in IL-1 α at 24 hours was not altered by T \times Hem or by alcohol binge alone, but was significantly suppressed (70%, $p < 0.05$) in cells obtained from alcohol-treated T \times Hem animals. No alterations in the magnitude of the IL-10 response were detected in any of the experimental groups.

DISCUSSION

The results from the present study show that 3-day alcohol binge decreased survival from T \times Hem, accentuated hemodynamic instability, attenuated neuroendocrine response, and blunted lung TNF- α expression immediately at completion of the fluid resuscitation protocol. Furthermore, the results from this study show that 3-day alcohol binge affects the integrity of the host response to a secondary inflammatory challenge during the recovery phase of traumatic injury through selective suppression of proinflammatory cytokines with a preserved anti-inflammatory cytokine response. These findings provide an insight into the mechanism of impaired host defense during the recovery period from traumatic injury during alcohol intoxication.

Three-day alcohol binge before T \times Hem decreased baseline blood pressure as well as the blood volume removed required to achieve similar hypotension as that achieved in dextrose-treated animals. Although alcohol-induced diuresis (Taivainen et al., 1995) could result in a decreased effective blood volume and thus decreased basal hypotension, no change in hematocrit was detected in alcohol-treated animals before initiating the T \times Hem protocol. The greater decline in blood pressure in response to a given blood loss could be the result of vasodilation (Brackett et al., 1994), decreased cardiac output (Malt and Baue, 1971), impaired vasoreactivity (Edgarian and Altura, 1976), or depressed myocardial contractility (Horton, 1992; McDonough et al., 1999; Thomas et al., 1989), all of which have been demonstrated to contribute to alcohol-induced changes in hemodynamics. Alternatively, it is likely that the alcohol-induced blunting of sympathetic and sympathoadrenal responses to blood loss could have played a major role in the decreased tolerance to blood loss seen in the alcohol-treated animals. This

suppression in neuroendocrine responses to blood loss in alcohol-treated animals has been consistently demonstrated by our studies investigating fixed-pressure as well as fixed-volume hemorrhage (Mathis et al., 2006; Molina et al., 2004a). Clearly, alcohol-induced impairment of these central neuroendocrine mechanisms involved in restoring blood pressure is likely to enhance susceptibility to tissue injury. In the present studies, however, skeletal muscle injury does not appear to have been aggravated, as evidenced by similar elevations in creatine kinase in alcohol-treated and dextrose-treated animals. Nevertheless, our previous studies have shown enhanced elevation of liver enzymes as well as greater base deficit at completion of hemorrhage and fluid resuscitation in alcohol-treated animals, suggesting that impaired hemodynamic counter-regulation to blood loss in alcohol-intoxicated animals does affect end-organ metabolism and likely contributes to tissue injury susceptibility (Phelan et al., 2002). Two physiological parameters, greater hypotension and metabolic acidosis at the time of entry into the emergency room, have been identified as critical determinants of outcome from traumatic injury (Heckbert et al., 1998; Miller et al., 2002). Thus, our results indicate that alcohol intoxication, acute or following a 3-day binge before or during traumatic injury, will most likely aggravate the outcome of injured victims. This is in agreement with clinical observations, rendering our model a clinically relevant approach to examine the mechanisms involved in impaired outcome from traumatic injury during alcohol intoxication (Dunham et al., 2000; Zehtabchi et al., 2004). Whether pharmacological manipulation to enhance sympathetic outflow or administer systemic vasopressors during the resuscitation period could ameliorate the hemodynamic instability resulting from alcohol intoxication is currently the focus of our studies.

In agreement with previous reports from our laboratory, T \times Hem produced an early induction of lung and spleen cytokine (TNF- α and IL-1 α) expression (Mathis et al., 2006; Phelan et al., 2002). Alcohol binge blunted the magnitude of the T \times Hem-induced increase in lung TNF- α but did not produce significant alteration of the lung IL-1 α response or of the spleen TNF- α and IL-1 α response. This is in contrast to our previous observations in animals that received a continuous 15-hour infusion in which tissue proinflammatory responses to hemorrhage were accentuated (Phelan et al., 2002) but similar to the effects produced by a single dose of alcohol administered before hemorrhage (Mathis et al., 2006). These apparently disparate effects of alcohol on immune responses have been attributed to differential duration of alcohol exposure, with acute alcohol resulting in most cases in suppression of proinflammatory responses (Boe et al., 2001; Szabo, 1999; Zhang et al., 2002) and chronic alcohol exposure favoring a proinflammatory response (Bautista, 1995; Diehl, 1998; Yang et al., 1998). Alternatively, it is possible that the altered neuroendocrine response in

alcohol-treated animals, particularly the attenuation in epinephrine and norepinephrine levels, could affect the magnitude of the T×Hem-induced changes in tissue cytokine expression. Several studies have established evidence of the neuroendocrine-immunomodulatory interactions, particularly that of the sympathetic nervous system (Webster et al., 2002). Our studies have shown that sympathectomy accentuates the hemorrhage-induced lung and spleen proinflammatory cytokine expression (Molina, 2001) and that alcohol intoxication during hemorrhage impairs the adrenergic-induced suppression in LPS-stimulated TNF release (Molina et al., 2004b). In addition, the sympathetic response to hemorrhage and tissue injury appears to be severely affected in alcohol-intoxicated animals as shown in this and our previous studies. Attributing the alterations in cytokine responses observed in the present studies (both in vivo and in vitro) to the alcohol-induced modulation of neuroendocrine response is not possible, given the condition-specific effects we have reported. Nevertheless, the possibility that the neuroendocrine mechanisms that control inflammatory responses to blood loss or to a challenge like LPS are deranged in the presence of alcohol is strongly supported by the findings of this and our previous studies.

In agreement with our previous findings, the increased MPO activity detected in the lungs of T×Hem animals was not altered by alcohol binge. Nevertheless, our previous studies indicate that even though neutrophil recruitment at completion of hemorrhage and fluid resuscitation is not altered by alcohol intoxication, the response to an infectious challenge is compromised, leading to impaired outcome from *Klebsiella pneumoniae* infection during recovery from trauma hemorrhage (Zambell et al., 2004) as well as following an intratracheal challenge with *Streptococcus pneumoniae* during alcohol intoxication alone (Boe et al., 2001). Thus, while no alteration in neutrophil recruitment at the post-T×Hem period may appear beneficial, the response to a “second-hit” challenge remains impaired, having a detrimental impact on the control of infectious processes.

Overall, it appears that the alterations in tissue cytokines observed immediately at completion of the T×Hem period in this model of alcohol binge are more modest than those seen in our previous studies and observed in the lung but not in the spleen. These findings were somewhat unexpected, as the animals had been exposed to alcohol for a longer period of time (3 days in contrast to 30 minutes or 15 hours) in our previous studies and, in addition, because the model of injury involved not only blood loss but soft tissue injury as well. Nevertheless, although prevailing tissue cytokine levels may not reflect severe compromise of host defense mechanisms, it is the response to a “second-hit” challenge during the recovery from traumatic injury that frequently leads to an increased incidence of infections and organ failure in trauma victims. Thus, we extended our studies to examine the initial recovery phase from T×Hem

and, specifically, to determine the integrity of compartmentalized cellular patterns of response and how these were affected by alcohol binge and T×Hem. Our results indicate that despite the modest alterations in the early proinflammatory cytokine response to T×Hem, alcohol binge had profound inhibitory effects on proinflammatory response elicited by in vitro LPS challenge, particularly in PBMCs and cells isolated from the pleural cavity, without affecting the LPS-induced IL-10 response. Similar anti-inflammatory effects of alcohol have been reported previously in PBMCs isolated from human volunteers 16 hours after drinking 2 mL vodka/kg (Mandrekar et al., 2006) as well as following surgical intervention in long-term alcoholic subjects (Spies et al., 2004) and these have been attributed in part to up-regulation of IL-10. The results from our studies do not show marked up-regulation of IL-10 in response to LPS stimulation in cells obtained from alcohol-treated animals. However, while attenuated TNF, IL-1, and IL-6 responses were noted in the cells obtained from alcohol-treated T×Hem animals, the IL-10 response was preserved, suggesting a shift in the balance of Th1/Th2 cytokine profile in favor of an anti-inflammatory state. Taken together, these findings and those from our previous studies showing greater mortality from infectious challenge during recovery from hemorrhagic shock in alcohol-intoxicated animals suggest that alcohol intoxication results in disruption of the Th1/Th2 balance during recovery from T×Hem, increasing susceptibility to a “second-hit” infectious challenge. Similar results and conclusions have been obtained in alcohol-fed mice infected with *K. pneumoniae* (Zisman et al., 1998). Interestingly, no significant changes were detected in response to LPS elicited in alveolar macrophages. These results would suggest differential effects of alcohol-binge and T×Hem on LPS-induced cellular responses that are not generalized to all immune cells and/or cellular compartments.

In contrast to previous reports in the literature demonstrating marked alterations in immune function and cellular responsiveness during the recovery period from T×Hem, our results did not show marked alterations in the LPS-induced cytokine responses examined 18 hours after completion of T×Hem and fluid resuscitation. This is most likely due to a more modest injury model used in the present studies. The combination of soft tissue injury and hemorrhagic shock used did not produce mortality during the injury period or during the initial recovery period. Nevertheless, other models of more pronounced tissue injury and hypotension (both in duration and magnitude) have clearly been demonstrated to produce marked and sustained immunosuppressive effects (Wichmann et al., 1998) as elegantly reviewed by Xu et al., (1998). Thus, it is important to note that alcohol binge before moderate T×Hem, which in itself does not result in sustained derangements in immune cell responsiveness, results in marked immunosuppression during the recovery period of surviving animals.

In conclusion, 3-day alcohol binge produces similar impairments in acute hemodynamic and neuroendocrine counterregulatory responses as does a single dose of alcohol. The immediate tissue T×Hem-induced cytokine responses appear to be relatively preserved, with the exception of a modest blunting of the hemorrhage-induced up-regulation of lung TNF. However, our results indicate that alcohol binge decreased survival from T×Hem and fluid resuscitation and furthermore, produced marked derangements in the cellular responsiveness to LPS stimulation during the recovery period in surviving animals. The blunted proinflammatory cytokine responses with preserved IL-10 responses suggest a shift in the balance of proinflammatory and anti-inflammatory mechanisms that is likely to be a central mechanism involved in the deranged host response to infectious processes during the recovery period. The observed immune-modulating effects of alcohol binge have direct clinical relevance and implications and suggest that alcohol-abusing trauma victims may benefit from immunomodulatory interventions aimed at decreasing their risk for infectious complications during the recovery period.

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